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James E. Trosko, Ph.D.

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FIELD	GROUP	SUB-GROUP	Meridian ACAS-570; Laser image analyzer; Quantitative fluorescence detection; Mechanisms of chemical toxicities

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This proposal was to have our current Meridian ACAS 570 laser cytometer upgraded with a 5 watt coherent laser, Olympus microscope with a CCD camera and video monitor, a mass data storage computer and dual detector system. This upgrade allowed us new dimensions of research potentials for studying mechanisms of chemical-induced toxicities in living cells, specifically enabling us to detect if chemicals caused toxicities by altering free Ca^{++} level, causing pH changes or induction of free radicals in single cells. During this year, we have pioneered the application of this new instrument in the demonstration that (a) heptachlor, a toxic pesticide/neurotoxicant, caused increased of free Ca^{++}; (b) several cytotoxic chemicals could generate free radicals; and (c) oncogene products could be detected using fluorescent antibodies; and (d) UV-induced DNA damage could be quantified using fluorescent antibodies.

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22 JAN 1990

TERMINAL REPORT AFOSR-TR- 90-0115

DURIP-Upgrade of the Meridian ACAS-470 for Toxicological Research

12/01/88 - 11/30/89

James E. Trosko, Ph.D.
Dept. of Pediatrics/Human Development
Michigan State University
East Lansing, Michigan 48824

I. Terminal Report of Progress

The purpose of this proposal was to upgrade our Meridian ACAS 470 laser cytometer, a novel and powerful interactive laser/computer assisted image analyzer. The device was designed to detect, using a microlaser beam, tracer fluorescent molecules in a cell and generate a false-color digitized image which could be quantitatively analyzed [see Appendix A]. This grant enabled us to bring this instrument up to the "state of the art", by allowing us to have a laser which could generate a powerful UV laser beam, as well as the visible laser beam we had. This gave us the potential to study molecules and mechanisms which could only be detected with UV-excitable dyes, as well as store and analyze more complex data, as well as manipulate the experiments with more precision.

The three original aims, i.e., (a) to detect intracellular free Ca^{++} [an indicator of both adaptive and maladaptive cellular physiology]; (b) to detect changes in intracellular pH with a pH sensitive dye (also a measure of both adaptive/maladaptive changes in cellular physiology caused by chemicals; and (c) to detect intracellular free radical production in single cells with a fluorescent dye caused by many toxic chemicals, were achieved

II. Results of Upgrade

The Meridian company modified our instrument within months after the grant was awarded. Because of this upgrade, we were able, in collaboration with the Meridian company, to develop several new applications with this new instrument leading to several manuscripts which are now in press or in preparation, as well as the development of new "Application Notes" [Appendix B].

III. Benefits to Research, Department and University

To re-iterate, since my USAFOSR grant [AFOSR-89-0325] is to study the mechanisms of chemical toxicants which act by inhibiting intercellular communication, the upgrade of this unique interactive laser image analyzer has made it possible to (a) do quantitative studies, on a single cell basis, which were either impossible or extremely difficult to do and inaccurate prior to its development; (b) increase our productivity by allowing us to generate data and to analyze data, simultaneously, without tying up the machine for one or the other; and (c) develop new potential applications for toxicological research [i.e., our recent use to detect specific oncogene products in normal or cancer cells].

As a result of the upgrade, both the number and diversity of studies using the Meridian ACAS-570 have increased. For example, the instrument is signed up for use almost every day, including many evenings and weekends. Free calcium studies are being done by scientists (Drs. Kalimi and Wilson) to study the effects of

hormone imbalance on calcium homeostasis in testicular tissue; the effect of anti-parasitic drugs is being studied in schistosomes by Dr. Bennett (Pharmacology and Toxicology Dept., MSU); oncogene detection with fluorescent antibodies in cancer cells by E. de Feijter (a fcreign scholar); stem-cell lineages in human breast tissue (Dr. S. Nakatsuka); DNA damage with fluorescent antibodies to UV-induced pyrimidine dimers by Dr. C.C. Chang (Dept. Pediatrics/Human Development); the reaction rates of enzymatic functions in bio-engineered matrices (Dr. M. Worden, Chemical Engineering Dept., MSU); a new way to measure heterologous intercellular communication (Dr. G. Kalimi, Dept. Pediatrics/Human Development) [see Appendix C].

In addition, benefit to the University accrues in many ways: (a) Collaboration with other research groups studying toxicology at different levels; for example, Dr. K. Honn of the Chemistry Department at Wayne State University has used our upgrade to initiate studies on the detection of free radicals after cells were exposed to eiconosoids; Dr. James Klaunig of the Medical College of Ohio in Toledo is collaborating with us to study chemical inhibition of intercellular communication in liver slices; and Dr. Phil Liu of Case/Western-Reserve University (Cleveland) is collaborating with us to detect DNA polymerases in single cells; (b) Because we were the first group using the Meridian ACAS and its upgrade, we are now communicating with others across the country and sharing new information at applications; (c) Because of the major technological breakthroughs represented by this instrument, I also have been asked to give workshops and seminars [i.e., At the latest Cell Biology meeting in Houston, Texas, I conducted a workshop].

Finally, this upgrade has also helped the University directly by attracting other grants utilizing this instrumentation [NSF grant to Dr. Worden in Chemical Engineering; NIEHS grant to Dr. L. Fischer, Center for Environmental Toxicology, MSU]. Several papers, abstracts and multiple speaking invitations, including one to Germany [International Gap Junction Meeting]; to England [International Society of Toxicology Meeting]; and recently to France [Workshop on Alternatives to Animal Toxicity Testing].

IV. Papers, Meetings and Seminars

1. J.E. Trosko and C.C. Chang, "Stem cell theory of carcinogenesis", Toxicology Letters, in press.

2. J.E. Trosko, "Towards understanding carcinogenic hazards: A crisis in paradigms", J. Amer. College of Toxicol., 8:1121-1132, 1989.

3. J.E. Trosko, C.C. Chang and B.V. Madhukar, "Cell-cell communication: Relationship of stem cells to the carcinogenic process", In: Mouse Liver Carcinogenesis:

Mechanisms and Species Comparisons, T.J. Slaga, Ed., Alan R. Liss, Inc., New York, in press.

4. M. El-Fouly, J.E. Trosko, C.C. Chang and S.T. Warren, "Potential role of the human Ha-ras oncogene in the inhibition of gap junctional intercellular communication", Molecular Carcinogenesis, 2:131-135, 1989.

5. J.E. Trosko, C.C. Chang and B.V. Madhukar, "In vitro analysis of modulators of intercellular communication: Implications for biologically based risk assessment models for chemical exposure", Toxicology In Vitro, in press.

6. S.Y. Oh, B.V. Madhukar, C.C. Chang, J.E. Trosko and E. Beyer, "Characterization of gap junctional communication deficient mutants from a HG-PRT deficient rat liver epithelial cell line", provisionally accepted to

7. A.W. de Feijter, J.S. Ray, C.M. Weghorst, J.E. Klaunig, J.I. Goodman, C.C. Chang and J.E. Trosko, "Correlation between the methylation status of the v-Ha-ras oncogene, gap junctional communication and tumorigenicity in rat liver epithelial cells", Molecular Carcinogenesis, accepted for publication.

8. B.V. Madhukar, J.E. Trosko, and C.C. Chang, "Chemical, oncogene and growth factor modulation of gap junctional communication in carcinogenesis". In: Cell Interactions and Gap Junctions. Vol. 1, N. Sperelakis and W.C. Cole, eds., CRC Presa, Boca Raton, FL, pp. 143-157, 1989.

Abstracts

1. B.V. Madhukar, S.Y. Oh, E. de Feijter and J.E. Trosko, "Inhibition of intercellular communication by toxic xenobiotic chemicals in vitro in a human epithelial cell system", Soc. of Toxicology, Atlanta, GA, Feb. 27 - March 3, 1989. The Toxicologist 9:4, 1989.

2. S.G. Lilly, E. de Feijter, C.M. Weghorst, C.C. Chang, B.V. Madhukar, M. El-Fouly, J.E. Trosko, and J.E. Klaunig, "Reduced gap junctional intercellular communication and tumorigenicity of rat liver epithelial F344-WB cells". Soc. of Toxicol., Atlanta, GA, Feb. 27-March 3, 1989. The Toxicologist 9:123, 1989.

3. J.E. Trosko, "Mechanisms of chemical and oncogene modulation of gap junction and communication during carcinogenesis", Workshop on the Molecular and Cell Biology of Gap Junctions, Irsee, Germany, July 18-23,

1989.

4. J.E. Trosko, C.C. Chang. and B.V. Madhukar, "In vitro analysis of modulators of intercellular communication: Implication to mechanisms of tumor promotion and to predictions c)otential tumor promoters". 2nd Intern. Conf. on Practical In Vitro Toxicology, Nottingham, U.K., July 23-27, 1989.

5. J.E. Trosko, J.E. Klaunig, B.V. Madhukar, C.C. Chang, E. de Feijter, and G. Kalimi, "Chemical and oncogene modulation of intercellular communication during carcinogenesis". Symposium on Molecular Cell Biology of Liver Growth and Function, Lake Placid, NY, Aug. 13-16, 1989.

6. J.E. Trosko, J.E. Klaunig, M. Yeager, A. Koestner, M. El-Fouly, J. Buboltz, B. Cool, ard C.C. Chang, "Correlation between the reduction of gap junctional communication and tumorigenesis in rat glial and liver epithelial cells containing the expressed neu oncogene". Society of Toxicology Meeting, Miami Beach, FL, Feb. 12-16, 1990.

7. A.W. de Feijter, M.W. Lieberman, and J.E. Trosko, "Up-regulation of a metallothionein-ras T24 fusion gene in rat liver epithelial cells is correlated with the down regulation of gap junction function". Society of Toxicology Meeting, Miami Beach, FL, Feb. 12-16, 1990.

8. R.J. Ruch, J.E. Trosko, B.V. Madhukar, P. Somani, and J.E. Klaunig, "Restoration of gap junction intercellular communication by lovastatin in WB-H-rasII in rat liver epithelial cells". Society of Toxicology Meeting, Miami Beach, FL, Feb. 12-16, 1990.

9. B.V. Madhukar, H. Hong Xu, B. Lockwood, and J.E. Trosko, "Inhibition of intercellular communication by environmental chemicals in rat Leydig cells in vitro". Society of Toxicology Meeting, Miami Beach, FL, Feb. 12-16, 1990.

10. W.J. Paradee, B.V. Madhukar, and J.E Trosko, "Mezerein inhibition of intercellular communication and activation of protein kinase C in human kidney epithelial cells". Society of Toxicology Meeting, Miami Beach, FL, Feb. 12-16, 1990.

11. G. Kalimi, J.E. Trosko, L.L. Hampton, S.S. Thorgeirsson, and A.C. Huggett, "Gap junctional intercellular communication in raf and raf/myc transformed rat liver epithelial cell lines". American Association of Cancer

Research Meeting, Washington, D.C., May 23-26, 1990.

Meetings Attended and Seminars Given

1. Invited seminar speaker, "Chemical modification of intercellular communication: Implications for nongenotoxic toxicity". Program in Toxicology seminar series, Rutgers University, New Jersey, March 8, 1989.

2. Invited seminar speaker, "Chemical promoters. oncogenes, and growth factor modulation of intercellular communication: A unified theory". Loma Linda, California, March 21, 1989.

3. Invited symposium speaker, "Inhibition of intercellular communication during radiation and chemical carcinogenesis. Radiation Research meeting, Seattle, Washington, March 27, 1989.

4. Invited workshop speaker, "Promotion as a factor in risk assessment". Carcinogen Risk Assessment, Society for Risk Analysis, Wash. D.C., April 4, 1989.

5. Invited symposium speaker, "Oncogenesis and abnormal intercellular communication and its implication to the cause and prevention of carcinogenesis", and "Clinical implications of intercellular communication on wound healing". Quebec Association of General Surgeons, Quebec City, Canada, May 4-5, 1989.

6. Invited seminar speaker, "Oncogenes, tumor promoters and growth factor inhibition of intercellular communication: Integrative theory of cancer". University of Hamburg, Germany, July 17, 1989.

7. Invited symposium speaker, "Mechanisms of chemical and oncogene modulation of gap junctional communication during carcinogenesis". Molecular Cell Biology of Gap Junctions Symposium, Irsee, Germany, July 18-23, 1989.

8. Invited symposium speaker, "In vitro analysis of modulators of intercellular communication: Implications to mechanisms of tumor promotion and to predictions of potential tumor promoters". Practical In Vitro Toxicology Symposium, University of Nottingham, England, July 23-27, 1989.

9. Invited symposium speaker, "Chemical and oncogene modulation of intercellular communication during carcinogenesis". Symposium on Molecular, Cellular Biology of Liver Growth and Function, Lake Placid, NY, Aug. 13-16, 1989.

10. Invited seminar speaker, "Oncogenes, tumor promoters, and growth factors: An integrated theory of carcinogenesis". Eppley Cancer Center, Univ. of Nebraska, Omaha, Sept. 28, 1989.

11. Invited seminar speaker, "Role of chemical inhibition of gap junctioral communication in pharmacological and toxicological effects of drugs". Bristol Myers, Syracuse, NY, Oct. 9, 1989.

12. Invited symposium speaker, "Modulated intercellular communication: Consequence of extracellular communication". Eiconosoids and Bioactive Lipids in Cancer and Radiation Injury, Detroit, MI, Oct. 11-14, 1989.

13. Invited seminar speaker, "Modulation of gap junctional communication: Pharmacological and toxicological implications". Park-Davis Warner Lambert Pharmaceuticals, Ann Arbor, MI, Oct. 17, 1989.

14. Invited seminar speaker, "A crisis in paradigms: The roles of genotoxic versus epigenetic mechanisms in toxicology". Environmental and Industrial Health Department, Univ. of Michigan, Ann Arbor, MI, Dec. 1, 1989.

APPENDIX A

Information on New Meridian ACAS 570 Upgrade

APPENDIX B

Application Notes on new techniques that the Upgrade of ACAS can perform:

1. Calcium measurements.

2. Free Radicals.

3. Reprint "In situ (6-4) photoproduct determination by laser cytometry and autoradiography".

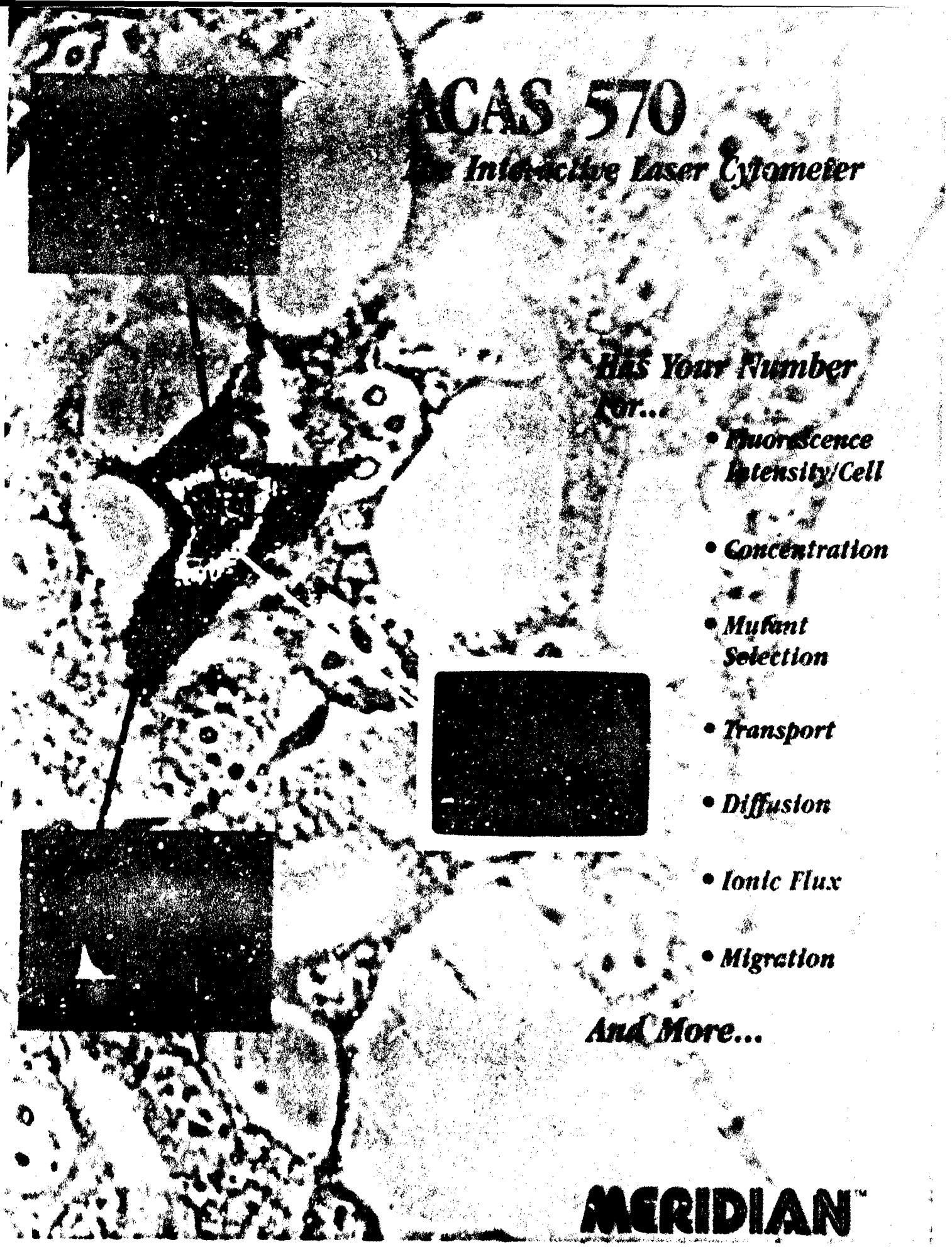
4. Reprint "Characterization of an in vitro human kidney epithelial system to study gap junctional intercellular communication".

APPENDIX C

New technique to measure heterologous and homologous cell-cell communication using the ACAS:

1. Actual ACAS-Image data.

2. Abstract.


ACAS 570
Interactive Laser Cytometer
Has Your Number For...
• Fluorescence Intensity/Cell
• Concentration
• Mutant Selection
• Transport
• Diffusion
• Ionic Flux
• Migration
And More...
MERIDIAN™

ACAS 570...

The Instrument for Quantitative Adherent Cell Analysis and Sorting

The ACAS 570 Interactive Laser Cytometer is uniquely designed for researchers who use quantitative fluorescence techniques to study adherent cells.

Conceived and developed by cell biologists for cell biologists, the ACAS — **Adherent Cell Analysis and Sorting** — provides fluorescence measurements of structure, function, and response at the single cell level. Applications range from cell and molecular biology to toxicology, immunology, and neurobiology.

Designed to insure you get the **numbers** you need, the ACAS 570 is the only instrument that provides capabilities to perform all of the following.

- Measure fluorescence distribution within cells
- Quantitate cellular responses using ratiometric methods for Ca^{++}, pH and other ions
- Measure macromolecular diffusion, permeation, and interaction
- Isolate and clone cells without removing them from culture
- Sort adherent cells based on physical and biochemical measurements
- Perform quantitative confocal imaging

Investigate these and other innovative applications that make the ACAS 570 — **The Cytometer for All Reasons.**

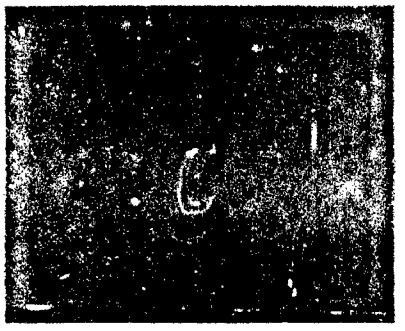
REASON • INTERACTIVE LASER CYTOMETRY

The ACAS 570 makes cytometry **interactive**

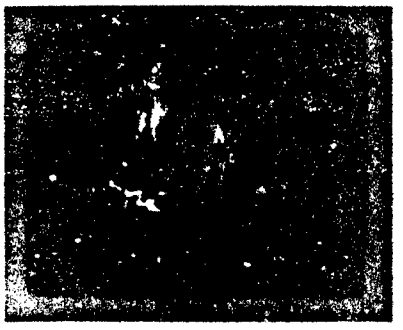
Only the ACAS lets you

- Isolate individual cells without removing them from their growth surface
- Create cellular fluorescence gradients by photobleaching or photoactivation
- Measure macromolecular diffusion or flux
- Monitor intracellular communications
- Perform microsurgery on cells

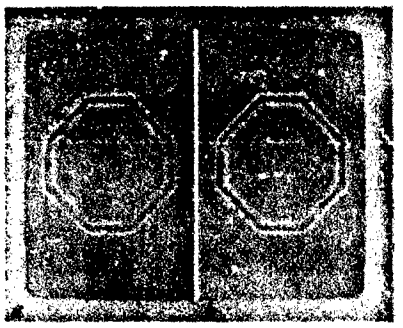
With the ACAS, you can use the laser for more than illumination. Now you can use the laser as a "photon gun," to photobleach or photoactivate for real-time measurements of cellular dynamics. Turn up the power and eliminate unwanted contaminating cells, or isolate and retain rare cells.



Two-Dimensional, pseudo-color fluorescent image of WI38 cell stained with the membrane potential dye $DiOC_6(3)$. (Courtesy of Dr. Bruce Jensen, Zynaxis Cell Science, Inc.)

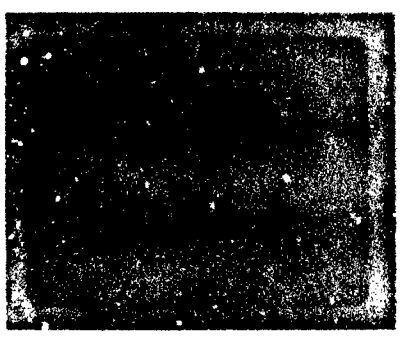


Intensity dimension plot of the membrane potential dye $DiOC_6(3)$ in WI38 Cell. (Courtesy of Dr. Bruce Jensen Zynaxis Cell Science, Inc.)



a. CHO cell isolated by the "Cookie Cutter"™ technique (Courtesy of Dr. Margaret Wade, Meridian Instruments, Inc.)

b. Cell outgrowth after two days



Fluorescence photobleaching of carboxyfluorescein diacetate labeled human teratocarcinoma cells as a measurement of cell-cell communication. Polygon enclosed cells are bleached and monitored for fluorescence recovery. (Courtesy of Dr. Margaret Wade, Meridian Instruments, Inc.)

Sorting Cells the ACAS Way

The ACAS is the only instrument that lets yo select anchorage-dependent cells directly fr heterogeneous cultures for isolation, propagation, and analysis.

The unique "Cookie-Cutter"™ technique automatically isolates single cells growing film-lined dish based on fluorescence measurements or morphology.

A high intensity laser beam encircles the sele cell, fusing octagon-shaped "cookies" to th culture dish. When the excess film is pulled the dish, the selected cell remains attached viable for continued propagation.

Use this powerful ***interactive*** technique f sorting:

- Mutant cells
- Transfected cells
- Hybridomas
- Rare cells for cloning

You can also use the ACAS laser as a "photo scalpel" to interact with cells grown on speci treated, heat-absorptive glass cover slips or culture dishes.

Command the laser to produce localized hea of target areas to:

- Select homogeneous cell populations
- Ablate single cells
- Eliminate large areas of cells

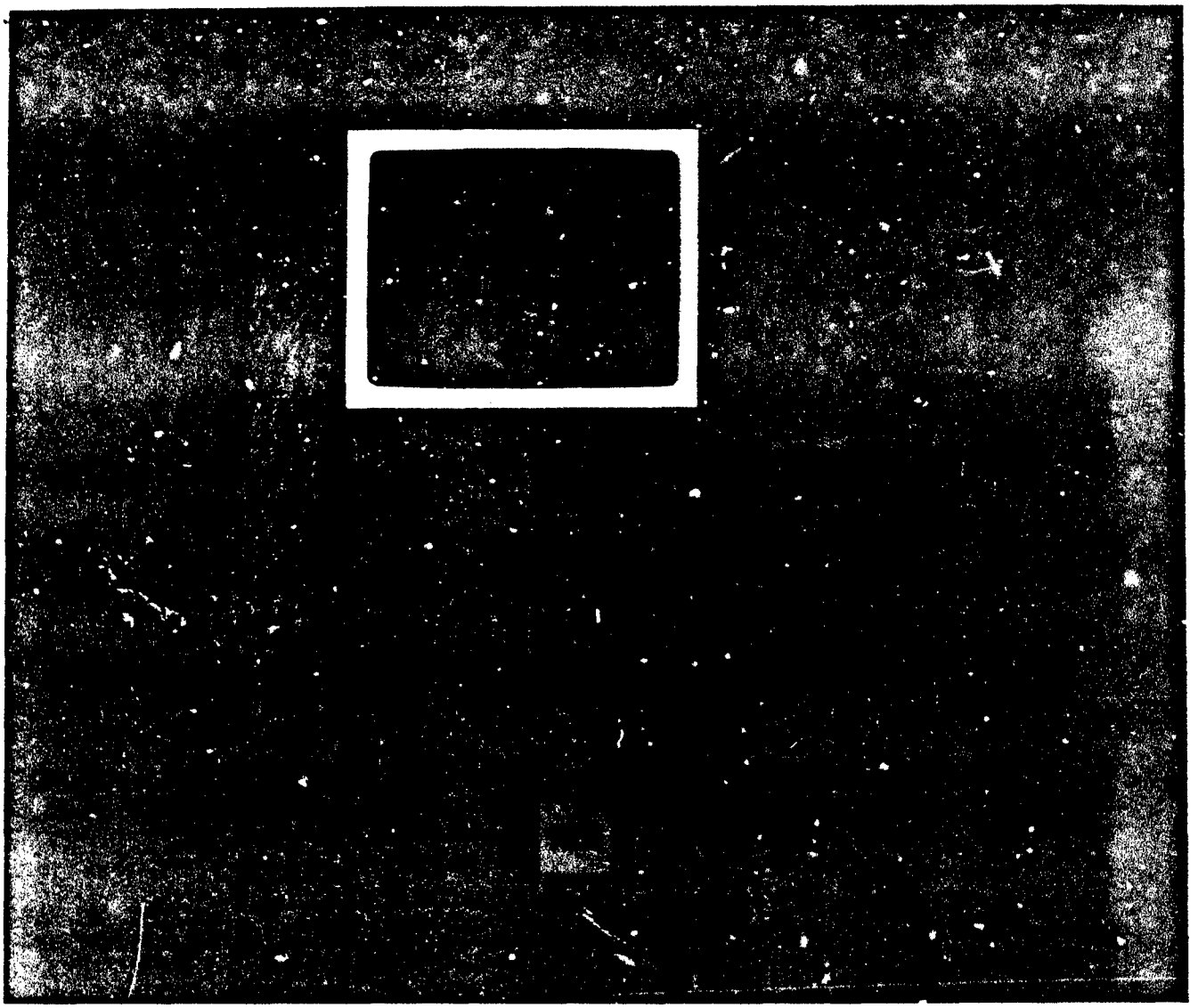
FRAP — Dynamic Parameters of Living Cells.

Fluorescence Redistribution After Photobleaching (FRAP) techniques involve photobleaching fluorescent dye molecules discrete sub-cellular areas with a high inte laser pulse creating non-fluorescent patches rate at which the patches are filled by unblea molecules from surrounding areas is meas by periodic, low intensity laser scanning.

Use this unique, non-destructive tool to di measure a wide range of cellular dynamics as:

- Receptor mobility
- Membrane transport
- Macromolecular assembly
- Cell-cell communication

"Cookie Cutter" is a trademark of Meridian Instruments, Inc.



From Signal to Image—The Basics

The schematic above shows you the basic optical and signal paths. The beam is directed along the center of the optical path and focused to a small spot by the microscope objective onto a fluorescently labeled cell.

Fluorescence emissions are collected by the objective, and directed to photomultiplier tubes by a series of mirrors and lenses. Appropriate wavelengths are selected by the choice of filters.

The following sequence describes how the ACAS acquires and displays fluorescence data:

1. Cells of interest are positioned above the objective by the X-Y scanning stage. A touch of the finger starts the stage moving to begin the scan.
2. As the stage moves, the focused laser beam is briefly pulsed by the AOM. Data is collected at intervals as small as 0.25 micron.
3. The photometric detection system captures the fluorescence emissions during the laser pulse. The resulting electronic signals are digitized and stored by the computer along with the stage coordinates.
4. The computer displays the signal resulting from a single laser pulse as a pixel with a color or gray value that represents the intensity of the emission at that point. Single or two dimensional scans are obtained by repeating this process while the state continues to move until the selected number of data points are acquired. (For example, see WISH cell on facing page.)
5. Fluorescence intensities are converted to numbers (i.e., concentration, rates of diffusion) depending on the software package selected.

REASON • QUANTITATIVE FLUORESCENCE MEASUREMENTS

Specifically designed for low level fluorescence detection and quantitation in living cells, the ACAS 570 gives you numbers you can count on.

Only with the ACAS can you:

- Measure physical and biochemical changes within cells
- Automatically acquire data and calculate histograms of fluorescence variations within cell populations
- Directly obtain macromolecular rates of diffusion
- Measure Ca^{++}, pH and other ions using ratiometric methods
- Obtain rapid (microseconds) temporal measurements

Sensitive, Quantitative Measurements with Minimal Photobleaching

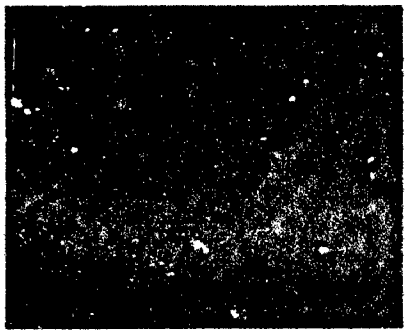
The ACAS 570 has harnessed the unique properties of coherent, monochromatic laser illumination to provide researchers with a powerful tool for fluorescence quantitation.

Fluorescent probes provide a high degree of sensitivity and specificity, but are susceptible to photobleaching, especially at the low concentrations required for the study of living cells.

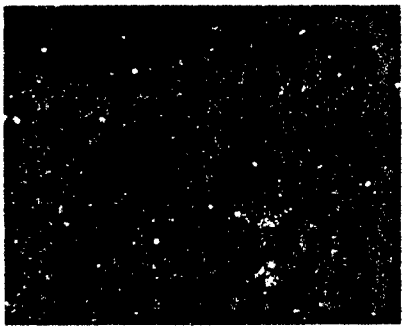
The ACAS is designed to resolve this problem through controlled laser intensity, duration, and spot size. The laser beam illuminates ***only*** the area to be measured, and ***only*** during the time of measurement.

A computer controlled acousto-optic modulator (AOM) provides rapid changes in the laser spot intensity and duration. Synchronized with the XY scanning stage, the AOM sequentially exposes small areas of a cell to brief (microseconds) laser pulses.

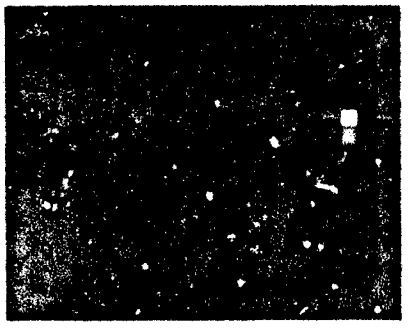
During a scan, therefore, each point in the field is illuminated for only a fraction of the time as required by full-field illumination systems, and with better sensitivity. The results **photobleaching under control.**



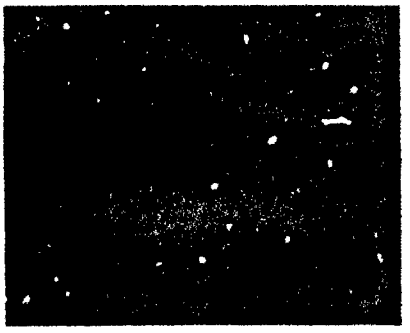
Serum stimulated increase in intracellular calcium levels in human teratocarcinoma cells labeled with Indo-1. (Courtesy of Dr. Margaret Wade, Meridian Instruments, Inc.)



Response of 3T3 cells labeled with the fluorescent pH indicator SNARF-1 to nigericin. (Courtesy of Asmina Jiwa, Meridian Instruments, Inc.)



Simultaneous dual label image of chick myoblasts stained with propidium iodide and fluorescent anti-creatine kinase. (Courtesy of Dr. LeAnn Quinn, Univ. of Washington School of Medicine.)



Detection of 30-60 copies of ABL mRNA by in-situ hybridization in the leukemia cell line K562. (Courtesy of Dr. Joel Greshoff, MD Anderson Hospital)

REASON • THE ACAS REVEALS THAN ME THE EYE

The ACAS uses a focused laser spot a advanced photometric detection, to sensitive, accurate fluorescence mea

With the ACAS you can:

- Minimize interference with cell functions by using lower dye concentrations
- Detect fluorescence even when small number of sites are label
- Perform multiple measurement cell with minimal photobleachi
- Simultaneously measure two er with the dual detector option

Photomultiplier tubes provide the sp gain, linear response, and large dyna not found in video-based systems. T under most experimental condition 570 provides you with reliable data cells with minimal photobleaching, without the need for signal averagir

With the ACAS 570, the On is Your Imagination.

From cloning cells to rapid calcium measurements, the ACAS Interactive Cytometer gives you more informati your cells than pretty pictures.

Designed by cell researchers, the AC integrates laser illumination, photo detection, and comprehensive, inte software, for the broadest range of a in cell and molecular biology avail single instrument.

Options such as quantitative confoca video processing, and dual photom offer you the flexibility and versatil accommodate present and future n

The ACAS also comes with the Meri commitment to total customer supp assuring maximum performance a productivity from your instrument. dedicated staff of cell biologists, eng computer scientists are immediatel with a phone call to answer your a and instrumentation questions.

These are a few of the many reason ACAS 570 is **The Cytometer For Reasons.**

To discover **YOURS,** call us at 1-800 In Michigan call 517-349-7200.

MERIDIAN™
Instruments, Inc.

The Cytometer for All Reasons

Tunable Argon-Ion Laser

Photometric Detection

Acousto-Optic Modulator (AOM)

Dual Monitors

Inverted Fluorescence Microscope

80386 Computer System

Keyboard and Mouse Interface

Motorized X Y Stage

your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(FAX) (517) 349-5967

Calcium Measurements in Single Cells with the ACAS Interactive Laser Cytometer

Margaret H. Wade, Ph.D. and Susan A. McQuiston, MT(ASCP), Meridian Instruments, Inc.

Intracellular free calcium in single cells can be quantitated using the ACAS Interactive Laser Cytometer. Small changes in free calcium can have dramatic effects on many cellular processes including cell motility, muscle contraction, gap junction function, fertilization and cell division (1-5).

Free intracellular calcium levels are typically between 50 and 150 nM. These levels are approximately 20,000 times lower than the total calcium concentration estimated to be bound in mammalian cells or in the external milieu. Most of the calcium within a cell is sequestered within nuclei, mitochondria, and endoplasmic and sarcoplasmic reticuli. Multiple mechanisms in the cell maintain this enormous concentration gradient. Other mechanisms are responsible for releasing free calcium ions into the cytosol in response to a variety of environmental stimuli (5,6).

Calcium ions are therefore regarded as "one of the most important intracellular messengers in biology" (7,8). Since the changes in free calcium levels which trigger cell activity are minute and often very rapid, quantitating these changes has been technically very difficult. Recently, however, Roger Tsien and his associates (9) have developed a fluorescent calcium indicator, Indo-1, which is particularly well suited for quantitating rapid calcium flux in single cells.

The fluorescent probe, Indo-1, is used to monitor calcium in its bound and free states by exciting the dye with the 351-363 nm lines of the argon laser, and simultaneously detecting two emissions at 405 nm and 485 nm (9). A ratio of these emissions is computed automatically by the integrated software and allows quantitation of free calcium within the cell. The ratio method eliminates most of the problems associated with dye leakage and concentration of fluorescent probe within the cell, and the simultaneous detection provides for extremely accurate quantitation of temporal and spatial calcium changes. The laser excitation source provides a small beam (about 1 micron in diameter) which both allows for extremely low levels of fluorescence to be detected with minimum laser power, and greatly reduces the amount of photobleaching that might occur with repeated scanning. The X-Y scanning stage allows for .25 micron data acquisition to provide maximum spatial analysis.

Several types of experimental data can be collected using the ACAS. Single point data, integrated cross sectional line scans as well as two dimensional spatial scans can be measured within a cell.

Calcium is quantitated directly in cells by comparing the ratio of the emissions to that generated in a standard curve. The standard curve is produced by mixing the Indo-1 free acid with varying amounts of added calcium in a physiological buffer containing EGTA. A typical standard curve is shown in Figure 1 where the Detector 2/Detector 1 ratio is plotted against free calcium concentration.

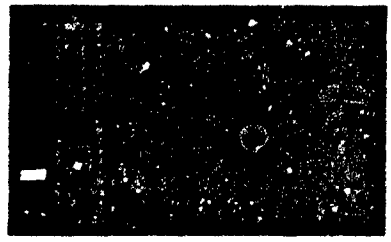

Figure 1. Standard curve of the ratio of the two emissions generated by adding calcium to EGTA containing solution of Indo-1 free acid.

Examples of line and image scan data are presented using serum starved human teratocarcinoma cells (HT) labelled with 1 uM Indo-1 AM, the cell permeant form of the dye. The addition of fetal calf serum to these cells causes a sharp 2-3 fold increase in calcium concentration which then returns to a baseline level. The particular component(s) in serum responsible for the calcium spike have not yet been identified.

Figures 2 and 3 are typical examples of line scan data. Figure 2 represents one of the consecutive scans across the cell. Figure 3 is a ratio plot of the integrated value under each scan as a function of time. The vertical mark denotes the time when serum was added. The temporal shift has occurred very rapidly and the calcium levels return to baseline.

Figure 2. Line scan data showing scans across the cell with fluorescence at both 405 and 485 displayed.

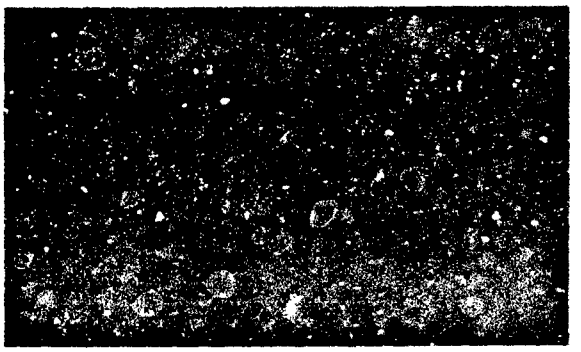

Figure 3. Ratio calculated from the integrated values determined from 400 line scans.

Figure 4-6 show an example of the 2-dimensional image scans that can be generated in a timed mode. The relative fluorescence intensity at both emissions in the first of twenty scans is plotted using the color scale denoted in the center of Figure 4. In Figure 5, serum was added, and by using the standard curve, the ratio of Detector 2 over Detector 1 is plotted as calcium values (calcium map). This map can be produced for each of the 20 images generated. In addition, a plot of the calcium concentration versus time in the designated cells can be produced as in Figure 6.

Use of Indo-1 provides high sensitivity of intracellular free calcium concentration. Controls should be used to insure that the fluorescence detected is calcium sensitive (9-12), including manganese quenching of the Indo-1 free acid, assessment of any Indo-1 ester fluorescence and polarity adjustments to the standard curve (13).

Quantitation of transient calcium values can easily be obtained in single cells using the ACAS Interactive Laser Cytometer. Spatial and temporal resolution of calcium can be analyzed using multiple scanning modes. Production of a standard curve allows for direct quantitation of calcium within cells.

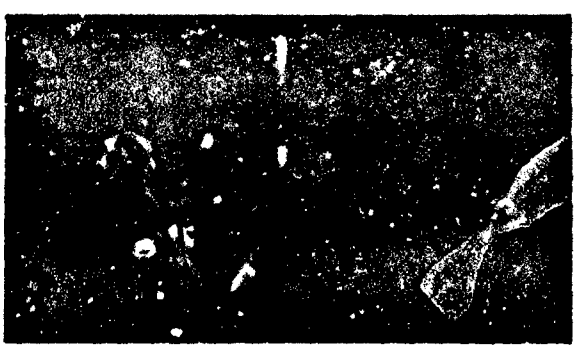

Figure 4 Two dimensional plot of data showing both emissions for a number of single cells.

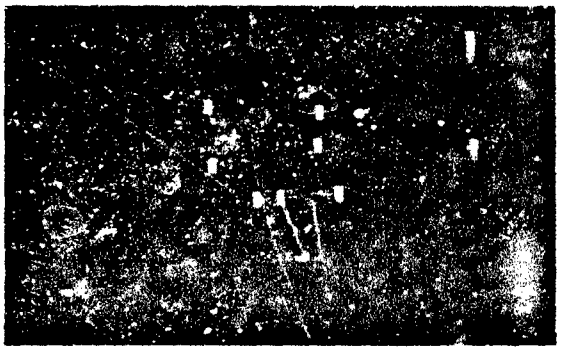

Figure 5. A plot of the ratio of detector 2/detector 1 after addition of serum.

Figure 6. Time plot of the ratio values expressed as calcium concentration from the cells in Figure 5.

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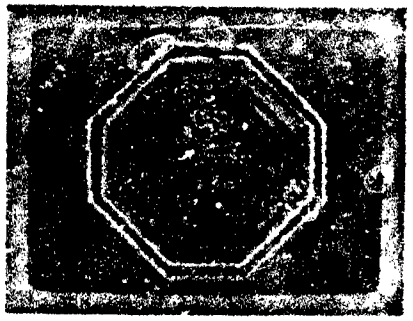
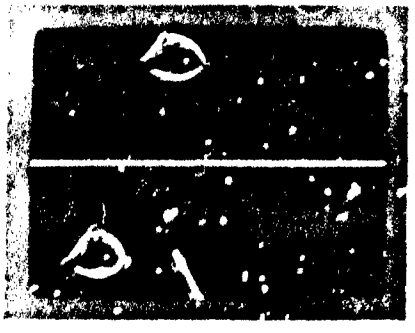


Outgrowth of CHO cell isolated by the "Cookie Cutter" technique (Courtesy of Dr. Margaret Wade, Meridian Instruments, Inc.)

Fluorescence photobleaching of carboxyfluorescein diacetate labeled human teratocarcinoma cells as a measurement of cell-cell communication. Polygon enclosed cells are bleached and monitored for fluorescence recovery. (Courtesy of Dr. Margaret Wade, Meridian Instruments, Inc.)

FRAP analysis of human fibroblasts stained with NBD phosphatidyl choline. Graph shows recovery of fluorescence following spot bleaching of the dot. (Courtesy of Dr. Margaret Wade, Meridian Instruments, Inc.)



Laser transection of PC-12 neurites. (Courtesy of Dr. Steven Heidemann, Michigan State Univ. Dept. of Physiology.)

ACAS 570...
The Cytometer for All Reasons

Reason #1 INTERACTIVE LASER CYTOMETRY

The ACAS 570 Interactive Laser Cytometer lets you go beyond observation to interaction with your cells in culture.

You command the power of the laser for new research capabilities including interactive techniques such as rare event selection, microsurgery, and fluorescence photobleaching.

CELL SELECTION IN CULTURE

The ACAS is the only instrument that lets you select cells directly from adherent cell cultures for isolation, propagation, and analysis.

• Sorting Cells the ACAS Way

Sort adherent cells without removing them from culture, using the unique, patented "Cookie-Cutter"™ process. This powerful automatically isolates a single cell based on analytical measurements (fluorescence, or morphology) from a population growing on a film lined dish.

A high intensity laser beam encircles the selected cells, fusing octagon shaped "cookies" to the culture dish. When the film is peeled off the dish, the selected cells remain attached and continue to propagate.

This powerful ***interactive*** technique is ideal when working with small quantities of cells and rare cells such as mutants, transfected, cells and hybridomas, even at frequencies of one in a million.

And with "cell-list" memory, your selected cell locations are stored for repeated scans of the same cell or population of cells over time.

• Laser Ablation

Isolate cell sub populations using automatic laser ablation to destroy either fluorescent or non-fluorescent cells, leaving desired cells undisturbed for continued growth as a homogeneous culture.

Cells are grown on specially treated glass cover slips which absorb high intensity laser light to produce localized heating and killing of only the target cells.

Cell Selection applications include:

- Mutant selection based on physical and biochemical characteristics including membrane protein lateral mobility, gap junction communication, and transfection
- Selection and cloning of transformed cell lines such as smooth muscle, Chinese hamster ovary, and human teratocarcinoma stem cells
- Isolation of subpopulations of human melanocytes, T cells, and parenchymal hepatocytes

CELLULAR MICROSURGERY

With the ACAS 570 you can use the laser as a "photon scalpel" to perform cell surgery on individual cells, create transient pores in cell membranes, and ablate sub-cellular organelles such as mitochondria and lysosomes. Laser surgery can be coupled with quantitative fluorescence analysis to provide insight into cellular structure and function.

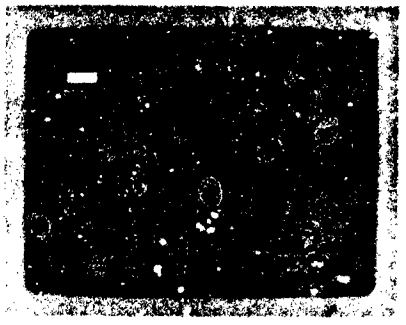
FLUORESCENCE PHOTOBLEACHING

The ACAS puts photobleaching under your control. Use the laser as a "photon gun" to photobleach or photoactivate for real-time measurements of molecular dynamics.

• Fluorescence Redistribution after Photobleaching (FRAP)

FRAP techniques involve photobleaching an area or volume of a fluorescently labeled cell with a high intensity laser pulse. The movement of unbleached fluorescent molecules from surrounding areas re-establishes fluorescence in the bleached zone.

Subcellular localization of protein kinase C in neuroblastoma-glioma (NG) cells using a fluorescent phorbol ester (Courtesy of Dr. H. A. Pershadsingh, Univ. of California, San Francisco)



Analysis of the distribution of sub cellular protein kinase C in 3T3 cells labeled with a fluorescent phorbol ester. Polygon enclosed areas are measured for distribution changes. (Courtesy of Dr. H. A. Pershadsingh, Univ. of California, San Francisco)

With the ACAS, you can directly measure molecular diffusion or flux, and degree of recovery, and use these rate determinations to explore cellular architecture and mechanics, including cytoskeleton organization, nuclear membrane structure, macromolecular assembly, and plant cell wall porosity.

• Cell-Cell Communication

Cell-cell communication techniques also rely on fluorescence photobleaching to measure intercellular communication between contacting plant or animal cells. Gap junction-mediated intercellular communication in animal cells is thought to play an important role in the regulation of cell proliferation and differentiation, providing a potential screening assay for environmental toxicants and drugs.

Use ACAS FRAP techniques to:

- Measure the lateral mobility of cell surface proteins
- Determine membrane potential
- Quantitate protein kinase C distribution and translocation
- Measure the modulation of gap junction-mediated cell-cell communication by tumor promoters, growth factors, and extracellular ions

Add the dimension of ***Interaction*** to your cell biology research. The ACAS 570 is the tool you need for cell selection and fluorescence measurements in culture.

This is only one reason why the ACAS 570 is the **Cytometer For All Reasons.**

For additional information call us at 800-[illegible] in Michigan call 517-[illegible] or [illegible]

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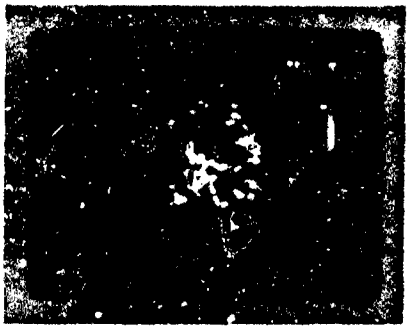
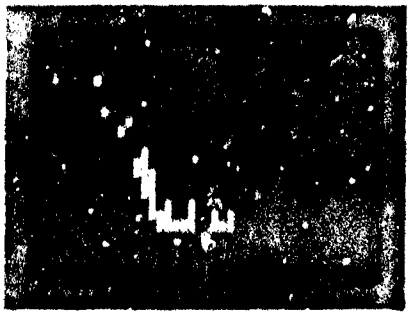
Calcium oscillations in freshly isolated rat aorta cells labeled with the fluorescent calcium probe Indo-1 (Courtesy of Dr. Oliver Flint, Bristol Myers Co.)



HIV-IRF infected CEM-SS cell stained by indirect immunofluorescence using anti-gp 120 (Courtesy of Dr. Jonathan Warren, National Institutes of Health)

Cholesterol ester accumulation in human macrophages stained with nile red from patients with coronary artery disease (Courtesy of Dr. Eugene Koren, Oklahoma Medical Research Foundation)



Histogram of cholesterol ester accumulation in human macrophages stained with nile red (Courtesy of Dr. Eugene Koren, Oklahoma Medical Research Foundation)

ACAS 570...
The Cytometer for All Reasons

Reason #2 **QUANTITATIVE FLUORESCENCE MEASUREMENTS**

The ACAS 570 Interactive Laser Cytometer unleashes the analytical power of fluorescence, giving you reproducible ***Quantitative*** measurements of cellular structure, function, and response.

From single and multiple measurements, to simultaneous emission ratios, you get ***real numbers*** for fluorescence quantitation ranging from DNA content and molecular diffusion, to intracellular Ca^{++} and pH.

QUANTITATIVE FLUORESCENCE APPLICATIONS

Physical and Biochemical Measurements

Using two dimensional fluorescence analysis you can easily identify and quantitate content, composition and distribution of cellular components such as lipoprotein, mitochondrial DNA, RNA, structural proteins, enzymes, and receptor molecules in one cell or hundreds of cells. In addition, you can measure a variety of biochemical activities such as membrane potential, activation states and ligand binding.

Current applications include:

- Quantitation of DNA, histone and cytoplasmic mass of blood cells
- Dynamic *in situ* distribution of protein kinase C using fluorescent phorbol esters
- Monoclonal marker identification of human tumor cells in mixed cultures
- *in situ* hybridization

• Calcium, pH and other Intracellular Ions.

With the ACAS 570 you can rapidly measure single, multiple or simultaneous dual emissions in a point, line or two dimensional image scan using fluorescent dyes such as SNARF-1™, Indo-1 and Fluo-3. Powerful software helps you generate a standard curve, and calculates ratio or dual label temporal and spatial concentration gradients.

Quantitative fluorescence measurements include:

- Cytosolic Ca^{++} response to a variety of chemicals, including tumor promoters, growth factors, and hormones
- Repetitive transient Ca^{++} oscillations in single heart muscle cells
- Simultaneous Ca^{++} and pH measurements using the visible probes Fluo-3 and SNARF-1

Fluorescence Redistribution after Photobleaching (FRAP)

The ACAS puts photobleaching under your control. Use the unique tool of fluorescence photobleaching to create optical gradients at specific intracellular sites or in whole cells for real-time measurements of cellular dynamics and patterns.

FRAP techniques directly measure molecular diffusion or flux, and degree of recovery, important parameters in understanding cellular mechanics and architecture.

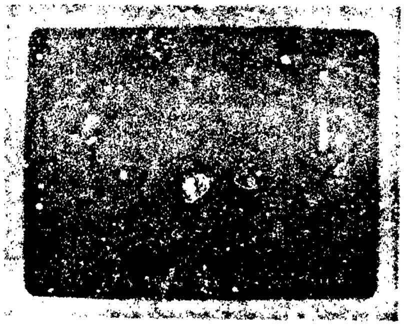
ACAS FRAP techniques measure:

- Lateral mobility of membrane proteins
- Cell to cell gap junction permeability
- Cell membrane macromolecular mobility rates

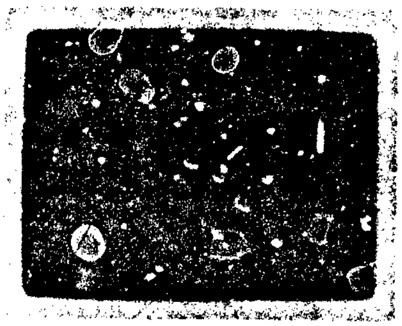
• Cell-Cell Communication

The ACAS is also used to measure intercellular communication between contiguous cells in animal and plant tissue.

This protocol, relying on fluorescence photobleaching, measures molecular transfer through cell gap junctions, gaining important insights into the regulation of cell proliferation and differentiation, and is a potential screening assay for environmental toxicants and drugs.



Detection of (6-4) photoproducts in UV-irradiated XP-A cells by a monoclonal antibody (UV dose = 0.1 W/m²) (Courtesy of Dr. Toshio Mori, Kanazawa University, Japan.)



Same as above except UV dose equals [illegible]

Intercellular communication measurements include:

- Inhibition of gap-junction mediated intercellular communication by tumor promoters and growth factors
- Modulation of gap-junction function by intracellular levels of Ca^{2+}, pH, and cAMP
- Control of transport between plant cells

• Quantitative Immunofluorescence

Immunofluorescence techniques can be used for sensitive, rapid screening assays on the ACAS. You can monitor antigen expression, cell binding and killing, as well as quantitative morphological information (i.e., mean fluorescence and cell size) for sensitive, non-subjective measurements.

Now you can use the ACAS for:

- Screening assays of anti-HIV compounds
- Cytotoxicity assay of NK cell binding to tumor cell targets
- Quantitative screening assays for antigen expression in tumor tissue sections

Add the dimension of ***quantitation*** to your [illegible] research. The ACAS 570 is the tool [illegible] for the measurement of molecular concentration, distribution and dynamics in [illegible] cells.

[illegible] the ACAS 570 is the **Cytometer For All Reasons.**

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ACAS 570...
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Reason #3 SOFTWARE THAT WORKS FOR YOU

The ACAS Interactive Laser Cytometer is ***the*** instrument for quantitative fluorescence analysis and adherent cell sorting.

The power of the ACAS lies in the comprehensive, interactive software that teams laser cytometry with advanced computer technology and a fast, flexible operating system — giving you integrated data acquisition, analysis and management capabilities that are easy to learn and easy to use.

APPLICATIONS BASED PROGRAMS FOR THE CELL BIOLOGIST

You get a powerful assortment of flexible programs with applications ranging from cell and molecular biology to toxicology, immunology and neurobiology.

- **Image Analyze** — Two dimensional fluorescence quantitation of single or dual emission.
- **Auto Image** — Automatic scanning and fluorescence quantitation of multiple fields in the same field over time.
- **Ratio Analysis** — Quantitation of intracellular Ca^{++} pH or other ions.
- **Cell Sorting** — Automated sorting of fluorescent cells.
- **FRAP** — Measurement of intramolecular diffusion.
- **Cell-Cell** — Measurement of intercellular communications via gap junctions or general kinetic measurements.
- **Cell List** — Store locations of selected cells for automated measurements, scanning, cell selection and reviewing.

SIMPLIFIED SYSTEM INTERACTION

Designed for simple operation right from the start, the intuitive, menu driven ACAS software helps you learn the system easily and become productive quickly. For example:

- Perform all operations from the logically organized main menu using easy pull-down menu functions.
- Use the convenient mouse or keyboard, working in parallel, for fast, simple interaction with instrument hardware *and* software.
- Follow the screen prompts and menu displays for instant selection of your next step.
- Custom tailor directories, analysis parameters, palette colors, graphics and many other programs with the integrated editor functions.

TOTAL CONTROL OVER YOUR EXPERIMENT

The ACAS software makes it easy to design and conduct your experiment directly from the screen by integrating optical, mechanical and computer hardware functions.

With the touch of a finger you can:

- Move the mouse arrow to easily select experimental parameters from the program menu.
- Make all computer controlled instrument functions such as movement of the scanning stage, and laser intensity and duration.
- Perform instrument calibration and system diagnostics from the computer screen.
- Automatically store cell locations for repeated scans of the same cells over time.

COMPREHENSIVE ANALYSIS FUNCTIONS

Use the mouse driven pull down menus to automatically perform a variety of image analysis and processing functions including:

- Correct, threshold and rescale data.
- Define cells with polygons for separate analysis.
- Eliminate cells for exclusion from final calculations.
- Query data from any area or at a specific point in an image.
- Create histograms based on cellular statistics (i.e. number of fluorescent cells, average fluorescence, area, perimeter and shape factors) integrating hundreds of experiments.
- Choose data files and preset parameters for automatic analysis.

POWERFUL GRAPHICS CAPABILITIES

Create a variety of analytical displays with the powerful graphics functions for accurate, in-depth visual interpretation of your data. For example:

- Display fluorescence scans as 256 gray-levels or various pseudo-color palettes.
- Illustrate population differences using multiparameter scatterplots and histograms.
- Overlay dual detector images for visual comparison and discrimination of fluorescence varies from each detector.
- Create composite displays of up to four screens for easy comparison of fluorescence images and corresponding graphics.
- Automatically create a graph of the fluorescence values along any specified cross section of a scan.

EASY, EFFICIENT DATA MANAGEMENT

The graphically designed ACAS software can input, store and retrieve your data with speed and flexibility.

- Perform disk and file maintenance quickly and easily with the dedicated ACAS file utility system.
- Use the mouse or keyboard to tag files for group or individual operations.
- Automatically locate specific files on any disk.
- MS-DOS operating system ensures compatibility with a wide range of other available software.
- Easily integrated into networks via Ethernet*.

* Ethernet is a trademark of Digital Equipment Corporation, Intel Corporation and Xerox Corporation.

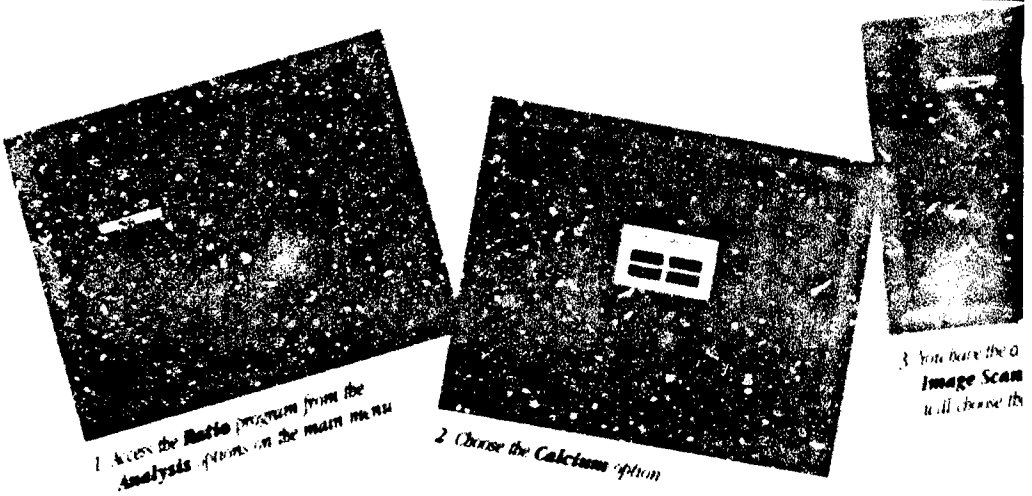
LET'S DO AN EXPERIMENT

The ease and simplicity of the powerful ACAS software can be shown best by following an experiment

So, let's examine the kinetics of calcium in living cells.

The ACAS 570 can be used to measure intracellular calcium in single cells or groups of cells. The dual wavelength **Simultaneous Detection System** provides ratiometric measurements for a variety of experiments. Single point scans, integrated cross sectional line scans, and two dimensional spatial scans can be measured.

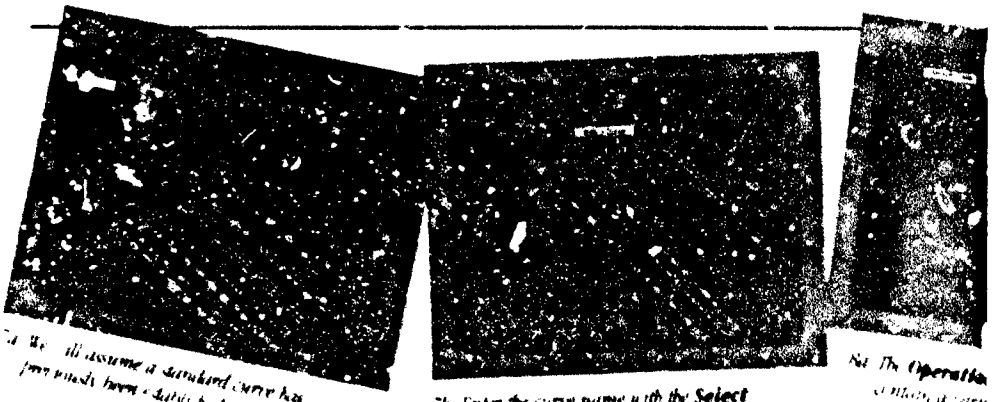
The integrated software automatically calculates intracellular calcium by comparing the emission ratios of the free and bound forms of a fluorescent dye such as Indo-1 to the ratios in a standard curve.



1. Access the **Ratio** *program from the* **Analysis** *option on the main menu*

2. Choose the **Calcium** *option*

3. You have the o **Image Scan** *will choose th*



a. We will assume a standard curve has [illegible] been established

b. Enter the curve name with the **Select Curve** *option*

Not The **Operatio** *[illegible]*

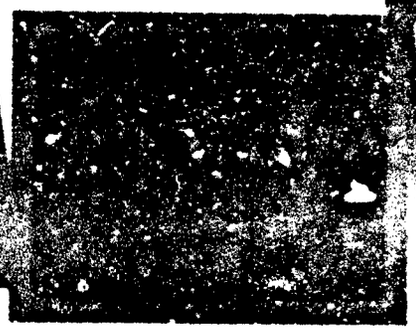
11. A two dimensional plot of the ratio of detector 2 detector 1 can be displayed using the **Map** *option. Data can also be* **Inverted** *from the map*

12. To calculate a pixel by pixel map of the Ca^{++} distribution as seen on the map, can be accomplished automatically

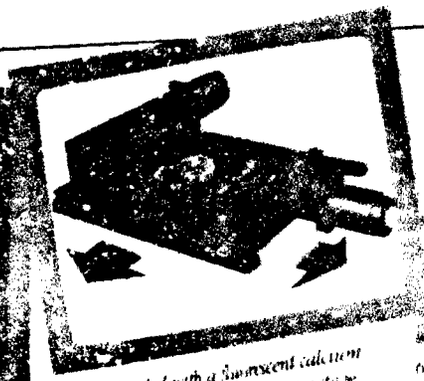
13. Graph of the change in Ca^{++} distribution over time for the marked areas can also be made automatically generated by choosing **Time Plot** *from the* **Operations** *menu*

14a. The **Show** *menu for* **[illegible]**

...ose the choice of a **Point Line** or ...ge **Scan** for this experiment or ... the **Image Scan**



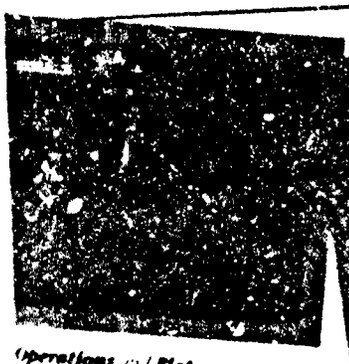
4 Begin the experiment by assigning a file name and setting instrument parameters for the scan from the options on the screen



5 Cells loaded with a fluorescent calcium probe are placed on the scanning stage. Use the mouse to manually move the stage and select a field of cells.

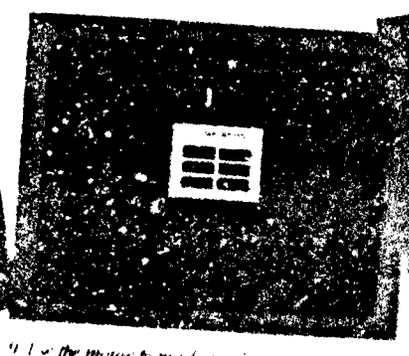
Begin scanning. Add the antagonist after the baseline Ca^{++} levels have been established. Continue scanning. Data is saved to the hard disk or removable cartridge disk.

6 To analyze this data, access **Image Analyze** from the **Analysis** program on the main menu and enter the file name.

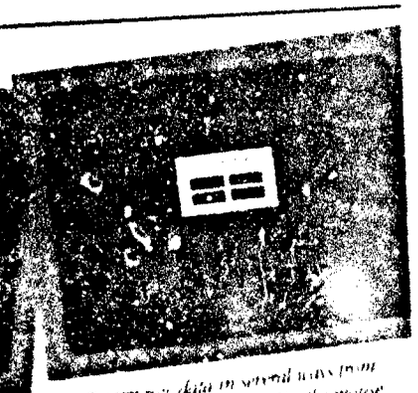


Operations or **Plot** menu ...
... options for data ...

... will find **Smooth Rescale** and **Threshold** ...



9 Use the mouse to mark ... cells with polygons for ... analysis.



10 **Query** ... data in several ways from the **Operations** menu. Use the mouse pointer to automatically report the calcium concentration within an entire scan area, within a polygon, ... cross section, or at a specific ...

... menu provides a variety of options ... to fit your ... needs.

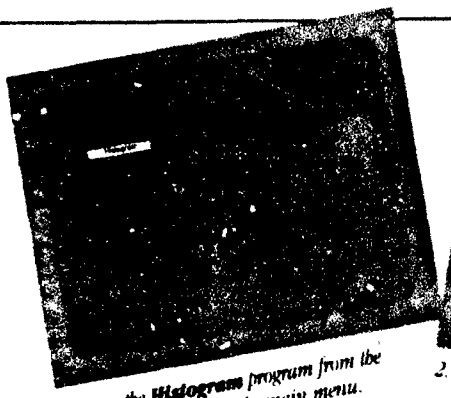
11 For example, **Multi plot** displays ... numerous ... of data ... and the time ... plot.

12 To end acquisition or analysis at ... time, use the **Control-Cancel** ... option always found on the menu.

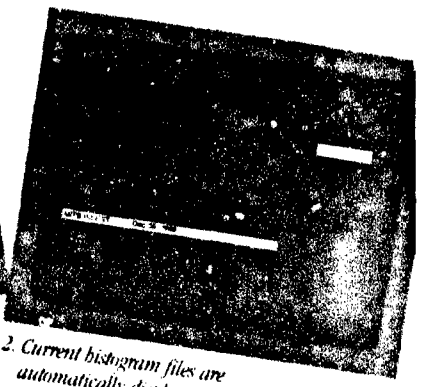
As you can see, there's nothing ... about our software. The unique design makes it easy to get the numbers you need for Calcium, and other cellular physical and biochemical measurements.

AUTOMATIC FILE INTEGRATION

Incorporating an innovative file utility system, the ACAS software automates data management for fast, simple file integration and statistical analysis. For example: you can easily integrate hundreds of individual experiments to create composite histograms and scatterplots illustrating a variety of analytical parameters.



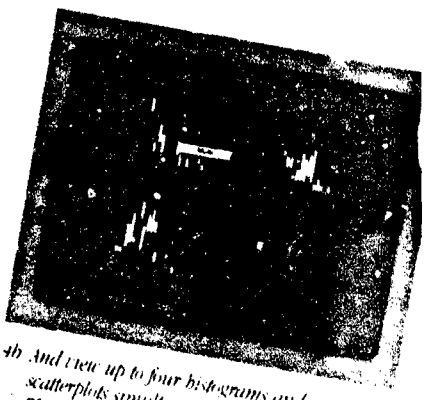
1. Enter the ***Histogram*** *program from the* ***Analysis*** *option on the main menu.*



2. Current histogram files are automatically displayed. Tag the histogram files you want for integration and analysis.

3. Choose histogram display parameters from the options listed on the ***Show*** *menu*

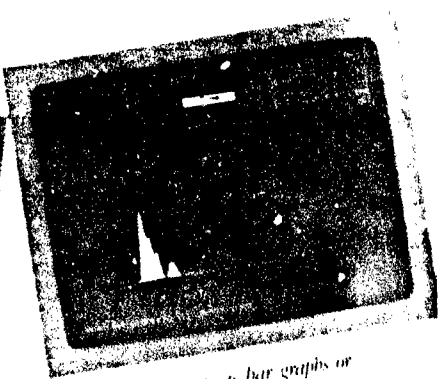
4a. You can also create dual parameter scatterplots.



4b. And view up to four histograms and scatterplots simultaneously from the ***Show*** *menu options.*

5a. The ***Operations*** *menu offers a variety of analysis and display options. For example,* ***Differentiate*** *assigns a specific color to individual files in the composite histogram for easy visual comparison*

5b. ***Window*** *gives you statistics on up to four areas (a window) of a histogram.*



6. Display histograms as bar graphs or curved plots, using the ***Plot*** *menu options. You can also export data to an ASCII format for further analysis*

These are just a few of the extensive data acquisition, analysis and management programs available with the **ACAS 570 Interactive Laser Cytometer**.

And, one more reason why the ACAS 570 is **The Cytometer For All Reasons**

To see more, and find out how the ACAS can work for **you**, call us at 1-800-247-8084, or (517) 349-7200.

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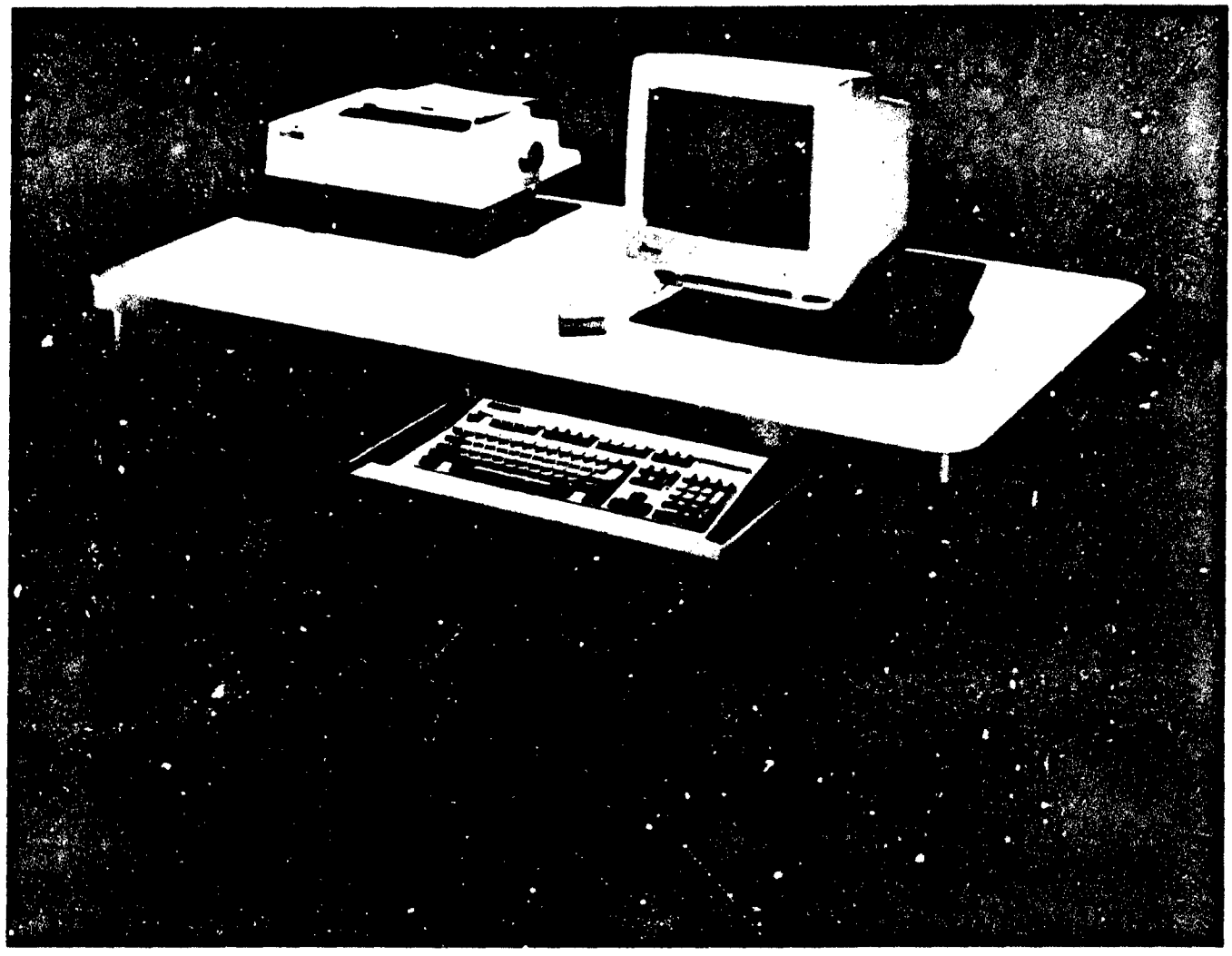
DASY 9000–Data Analysis System

Extend and Enhance the Analysis Capabilities of Your ACAS



- Improves Laboratory Productivity
- Duplicates ACAS 570 Analysis Capabilities
- Provides Convenient Access to Data
- Intuitive, Menu-Driven Software, Easy To Learn And Use
- Convenient Mouse and Keyboard Control
- Comprehensive Graphics Functions
- High Resolution Gray-Scale or Dynamic Color Displays
- High Quality Hard Copy Reproduction From Any Screen
- MS-DOS Disk and File Maintenance

The **DASY 9000 — Data Analysis System** — is a complete data analysis workstation dedicated to the analysis and display of fluorescence data gathered on the ACAS 470 or 570 Interactive Laser Cytometer. As an independent computer workstation, the DASY provides convenient access to data, and rapid execution of analysis programs without interrupting data acquisition on the ACAS.

You also get all the power of the comprehensive, versatile ACAS software, providing analysis, graphics and data management capabilities for a wide range of cell biology applications.

Now you can collect your data on removable, 20 Mbyte micro-bernoulli disks, or 1.2 Mbyte floppy disks for analysis on the DASY at any convenient time or location.

By relieving the ACAS of data analysis and reserving it for data acquisition, the DASY expands laboratory efficiency, saving you time and improving your productivity.

SPECIALIZED PROGRAMS FOR QUANTITATIVE FLUORESCENCE ANALYSIS

Specifically designed for cell and molecular biology applications, the DASY duplicates the powerful analytical capabilities of the ACAS giving you accurate, in-depth analysis of *DI* (*DNA*) adherent cell fluorescence measurements.

- Calculate fluorescence distribution within cells
- Quantitate cellular responses ratiometrically for Ca^{++}, pH and other intracellular ions
- Calculate molecular diffusion across cell membranes (FRAP, Cell-Cell communication)
- Characterize cell populations based on physical and biochemical measurements

VERSATILE SOFTWARE SIMPLIFIES INTERACTION

Designed for simple operation right from the start, the intuitive, menu-driven ACAS software helps you learn the system easily and become productive quickly.

All operations are performed with simple commands accessed by program selections from the main menu. Programs are logically organized and quickly chosen from pull-down menus using a keyboard or two-button mouse, working in parallel, to provide fast, easy system interaction.

For example: Use the mouse and pull-down menu operations to automatically perform a variety of image analysis functions including:

- Smooth and threshold data
- Mark selected areas for separate analysis
- Define cells with polygons for automated analysis
- Eliminate cells for exclusion from final calculations
- Calculate cellular statistics (i.e., number of cells, average cellular fluorescence, etc.)

POWERFUL ANALYTICAL GRAPHICS ILLUSTRATE FLUORESCENCE MEASUREMENTS

The comprehensive ACAS software gives you the capabilities to create detailed graphics for a variety of analytical displays.

- Automatically create histograms based on cellular statistics integrating hundreds of experiments
- Illustrate population differences (i.e., dual-detector scans) using multiparameter scatter plots, and bar or contour histograms
- Create composite displays of fluorescence images and corresponding graphics analysis for easy comparison
- Overlay dual detector images for visual comparison and discrimination of fluorescence values for each detector
- Query data from any area or at a specific point in an image

HIGH RESOLUTION COLOR IMAGES ENHANCE SIGNIFICANT DATA

Sharp, 640 × 480 pixel resolution gives you clear, vivid displays of contrasting cellular components and histogram populations.

Fluorescence scans are displayed as gray-scale or pseudo-color images composed from a variety of user defined color palettes.

High quality hard copies of any screen can be quickly obtained from the color printer, or through photographic reproduction via an optional in-line camera system for 35 mm slides and prints.

EASY, EFFICIENT DATA MANAGEMENT

Designed around a 32 bit, 80386 based microcomputer (IBM AT* compatible), the DASY can input, store, and retrieve your data with speed and flexibility.

Optimal program and data storage is achieved with a 60 Mbyte winchester hard disk and a 1.2 Mbyte floppy disk drive. (Additional storage capacity is available with an optional 20 Mbyte micro-bernoulli disk drive.)

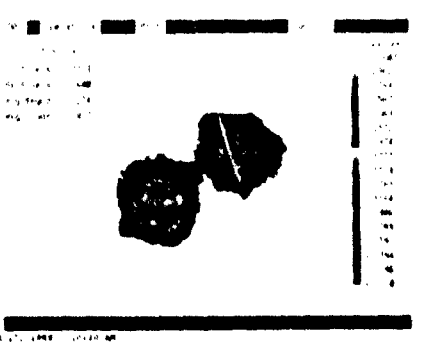
- **MS-DOS Operating System**

By utilizing the widely accepted MS-DOS operating system, the ACAS software provides simple, easy to learn programs, automating data processing and analysis, and minimizing data management time. MS-DOS also permits the use of a variety of commercially available programs and easy integration into local area networks via Ethernet**.

To find out how the DASY 9000 can increase the analytical power of ***your*** ACAS, call us at 800-247-8084. In Michigan call 517-349-7200.

Two dimensional pseudo-color fluorescent image of bladder tumor cell line MGH-U1 stained with rhodamine 123. Highly fluorescent areas represent regions containing mitochondria surrounding the less fluorescent nucleus. (Courtesy of Dr. Chi Wei Lin, Massachusetts General Hospital and Harvard Medical School)

Two dimensional gray-scale fluorescent image of the bladder tumor cell line MGH-U1, stained with rhodamine-123.



Color printer reproduction of two-dimensional pseudo-color fluorescent image of bladder tumor cell line MGH-U1 stained with rhodamine-123.

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Rev. 12'06'89

APPENDIX B

Application Notes on new techniques that the Upgrade of ACAS can perform:

1. Calcium measurements.

2. Free Radicals.

3. Reprint "In situ (6-4) photoproduct determination by laser cytometry and autoradiography".

4. Reprint "Characterization of an in vitro human kidney epithelial system to study gap junctional intercellular communication".

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Application Note Number E-2
Calcium Analysis

DETECTING CALCIUM RESPONSE IN CULTURED CELLS USING THE VISIBLE WAVELENGTH CALCIUM PROBE, FLUO-3

Margaret H. Wade, Meridian Instruments, Inc., Okemos, MI.

The measurement and quantitation of intracellular calcium responses within cells is of great importance to cell biologists (see Application Note E-1). Small changes in free intracellular calcium can effect gap junction function, cell division, muscle contraction, neutrophil stimulation and other cell functions. Recently several fluorescent probes to measure calcium have been developed by Roger Tsien. Indo-1 and Fura-2 were developed as dual emission or dual excitation calcium sensitive probes, and more recently the single excitation and emission probes, Fluo-3 and Rhod-1 have been made available (1,2).

Fluo-3 is a new fluorescent probe which can be excited by the 488 nm line of the argon laser, and emits in the visible spectrum similar to fluorescein (520 nm). The advantage of this probe is that UV excitation is not required; however a major disadvantage is that it is difficult to quantitate the absolute calcium levels in cells because it is not possible to use a ratio technique. A calcium response, however, is relatively easy to detect in cells, and further quantitation could be done with the calcium ratio probe, Indo-1. Fluo-3 also offers the possibility of dual labeling experiments where one may wish to monitor another property of the cell simultaneously with calcium levels, such as pH or membrane potentials.

A solution of the Fluo-3 free acid in high potassium buffer containing 1 mM EGTA, pH 7.45 was examined using the ACAS 570 Interactive Laser Cytometer. The sample was excited with 488 nm light from the argon ion laser, and the emission above 515 nm was detected with the photomultiplier tube and digitized. The absolute fluorescent units are plotted in Figure 1 as a function of free calcium (Orion standard) calculated from total calcium using the Kd of EGTA (105 nM) in this buffer. There is clearly a relationship between added calcium and increased fluorescence. However, as the fluorescent value in cells may depend upon dye leakage, photobleaching, degree of labelling, etc., this type of calibration curve cannot be used for quantitation.

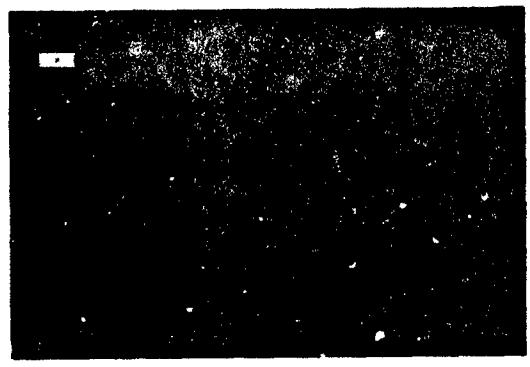

Figure 1. Standard curve showing Fluo-3 free acid fluorescence as a function of calcium concentration.

Human teratocarcinoma cells (HT) or normal diploid fibroblasts were grown in 35 mm tissue culture dishes and labelled with the cell permeable form of the dye, Fluo-3 AM, in PBS containing magnesium and calcium. Although the manufacturer suggests the addition of the solubilizing agent, pluronic acid (3), to the incubation media, we found this addition unnecessary for these cells (see Table 1). After rinsing, the dish was placed on the X-Y scanning stage and either a fluorescent image of a field of cells or a line scan across one cell was generated as a function of time. In some experiments, ionomycin was added and the calcium response was measured.

The concentration of Fluo-3 necessary to label cells was examined (Table 1). Cells incubated with 1 uM Fluo-3 were very poorly labeled and not included in the figure; those incubated with 3 or 5 uM for 1 or 2 hours showed good labelling without .05% pluronic acid, and less labelling with pluronic acid. We chose 5 uM Fluo-3 for 1 hour with no pluronic acid to perform the remaining experiments.

Average Fluorescence of Fluo-3

	3 uM	5uM
1 hr	285	1190
1 hr +Pluronic	212	598
2 hr	254	821
2 hr +Pluronic	127	703

Table 1. Average fluorescence per cell after various labelling conditions.

The photobleaching characteristics of cells labelled with Fluo-3 were determined by repetitively scanning the same area of the cells and plotting the integrated value as a function of scan number. Less than .1% fluorescence per scan is lost when using the ACAS (data not shown). This is a result of the use of a focussed laser beam (~1 micron in diameter) and Acousto-Optic Modulator (AOM). The AOM pulses the laser such that the sample is only illuminated for a brief period of time over a discrete area.

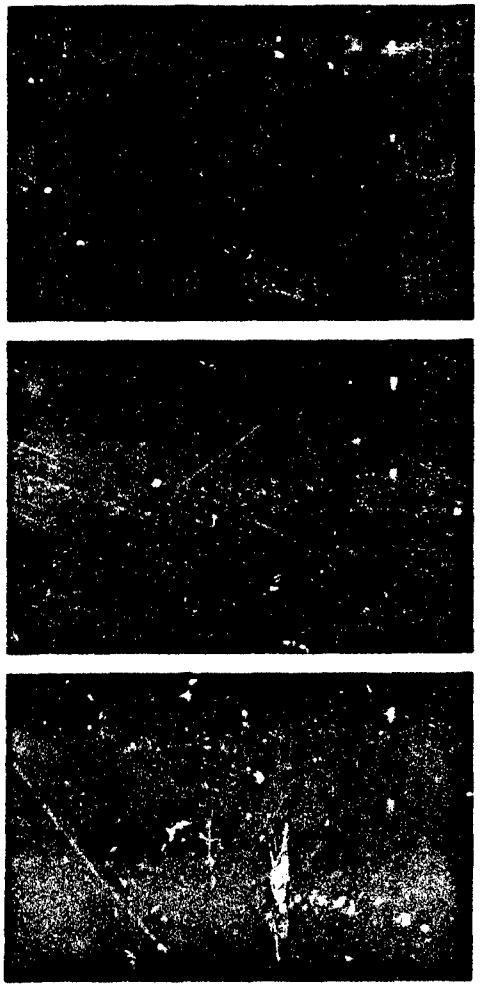

Figure 2. Two dimensional images of Fluo-3 incorporation in normal diploid fibroblasts A) before ionomycin B) immediately after ionomycin and C) 4 minutes after ionomycin.

The response of cells to the ionophore, ionomycin, is shown in Figures 2 and 3. HT cells were labelled with Fluo-3, and after the second image scan, 3 uM ionomycin in PBS containing calcium and magnesium, was added while the scanning continued. Figure 2a is a two dimensional pseudo-color image of normal diploid fibroblasts before ionomycin is added, Figure 2b is the same field immediately after ionomycin, and Figure 2c is the same field about 4 minutes later when the cells have returned to baseline values. Figure 3 is the time plot of a similar experiment with HT cells showing the time response to ionomycin. It is clear that an increase in fluorescence was detected, with a gradual return to baseline values. No attempt was made to correlate these fluorescent values with absolute calcium values.

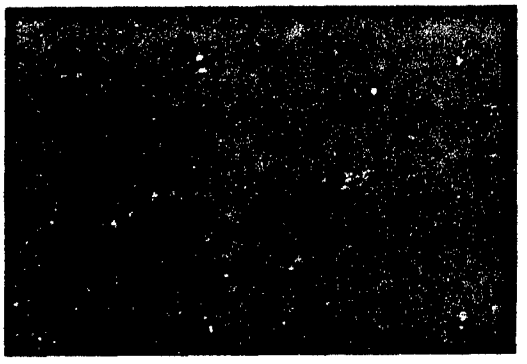

Figure 3. Time plot of ionomycin response in HT cells.

The new calcium sensitive probe, Fluo-3, will be very useful for determination of qualitative calcium changes in cells as the result of agonist treatment, such as growth factors, antibodies, etc. Although various methods have been suggested for quantitating calcium based on a fluorescent signal (3), one will always be concerned about the effect of dye leakage, photobleaching of the signal, unequal labelling among cells, etc. Fluo-3 is particularly useful as a first approximation of a calcium response because it uses visible, fluorescein-like, excitation and emission, and is suitable for simultaneous measurements of calcium and another fluorescent probe, i.e. pH.

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2310 Science Parkway
Okemos, Michigan 48864
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Detection and Quantitation of Peroxides and Hydroperoxides in Attached Cells Using the Dye 2,7-Dichlorofluorescin

David W. Bombick, R. J. Reynolds Tobacco Company, Winston-Salem, NC 27102

INTRODUCTION

Several active oxygen species are important in affecting biological systems. Peroxides and hydroperoxides (e.g. hydrogen peroxide) may form free radicals (such as the hydroxyl radical) which can react with many bio-organic compounds incuding proteins, nucleic acids and lipids. Since some of these reactions are harmful, measurement of active oxygen species can be important in toxicological research in areas including genetic toxicology, *in vitro* toxicology, immunotoxicology, reproductive toxicology and neurotoxicology.

Methods to quantitate hydroperoxides and peroxides in living cells are limited at the present time. Most techniques require cell-free systems and involve chromophores generated by reactions of thiobarbituric acid with malondialdehyde (1,2) or I^- with the peroxide (3). An assay to detect hydroperoxides based on the conversion of 2,7-dichlorofluorescin to the fluorescent 2,7-dichlorofluorescein by hydroperoxides has recently been developed (4,5,6). This reaction was sensitive to picomole levels and linear with respect to varying concentrations of a number of hydroperoxides and peroxides. The chemical 2,7-dichlorofluorescin diacetate, which freely crosses cellular membranes was used to facilitate loading into cells. Cellular esterases cleave the acetate groups and the ionic 2,7-dichlorofluorescin, which cannot cross cellular membranes, is trapped in cells. Once the 2,7-dichlorofluorescin is trapped within a cell it is available for oxidation to 2,7-dichlorofluorescein by the peroxides or hydroperoxides present. This method has been adapted for use with the Meridian Instruments ACAS 470 Interactive Laser Cytometer™ to allow quantitation of peroxides and hydroperoxides on a single cell basis.

METHODS

The dye 2,7-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) was dissolved in ethanol at a concentration of 1 mM. The dye was loaded into cells (5 ul/ml) in culture media for 30 minutes at 37° C. Chemical treatment of the cells may precede, follow, or occur simultaneously with the loading of the dye as long as this is kept constant in the experimental design and the treatment does not inhibit esterase activity. After 30 minutes of dye exposure, the plate of cells is rinsed with PBS containing 5 mM glucose (PBSg) approximately five times. The cells are maintained in a small amount of the PBSg (1-2mls) and examined with the ACAS 470 Interactive Laser Cytometer. The instrument is equipped with an argon ion laser tuned to 488 nm, and emission above 515 nm is quantitated from two dimensional image scans generated by a 1 micron laser beam and an X-Y scanning stage. An average fluorescence value from a confluent cell area or the fluorescence from single cells can be obtained. A standard curve (Figure 1) can be generated by adding varying concentrations of hydrogen peroxide (e.g., 10 ng/ml to 1 mg/ml) in PBSg with or without a confluent cell monolayer, depending upon the experimental design. This standard curve can be used to express fluorescence values as a function of hydrogen peroxide quantity.

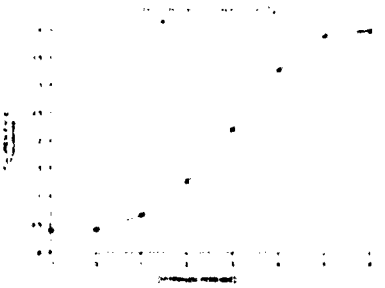

Figure 1. Standard curve showing hydrogen peroxide concentration (log ug/ml) as a function of fluorescence intensity.

RESULTS AND DISCUSSION

Quantitative analyses of peroxides and hydroperoxides in several cell types have been done using 2,7-dichlorofluorescin diacetate and the ACAS 470. Figure 2 illustrates the respiratory burst of neutrophils after addition of 10 nm/ml of PMA (phorbol-12-myristate-13-acetate). The cells were plated in multiple

dishes at equal densities and the average fluorescence values for neutrophils was calculated. At 0, 1, 5 and 10 minutes after PMA treatment those values were 305, 430, 931 and 1551, respectively. These values can be related to hydrogen peroxide concentrations with a standard curve. The rapid increase in peroxide formation parallels results obtained with flow cytometry (5). The grey areas within some of the images indicate the presence of cells which have not responded and are therefore not fluorescent. The images from the ACAS 470 demonstrate that the response is heterogeneous among the neutrophil population and that not all cells are activated to the same degree. This also demonstrates the importance of analyzing single cells, rather than a whole population. Figure 3 illustrates the fluorescent images generated when WB rat liver oval cells are exposed to a 12.45 mM concentration of hydrogen peroxide for 5 minutes. The conversion of 2,7-dichlorofluorescin to the fluorescent 2,7 -dichlorofluoresein in these cells is rapidly increased when hydrogen peroxide is exogenously added. Again, the response in the WB cells is heterogeneous which may indicate different subpopulations with varying protective mechanisms against hydrogen peroxide.

This method appears useful in determining whether particular toxicants generate peroxide or hydroperoxide species. Formation of these reactive oxygen species may be correlated to cytotoxicity parameters or other toxic manifestations. A further use of this method may include the examination of lipid peroxidation and subsequent membrane damage. Finally, an *in vitro* assay could be developed to test a chemical's anti-oxidant capability.

In summary this report discusses a method to quantitate the formation of reactive oxygen species *in vitro*. The advantages include the capability of carrying out the analyses in attached monolayers of cells rather than cell suspensions. In addition, detection of these peroxides and hydroperoxides can be done quickly and on a single cell basis.

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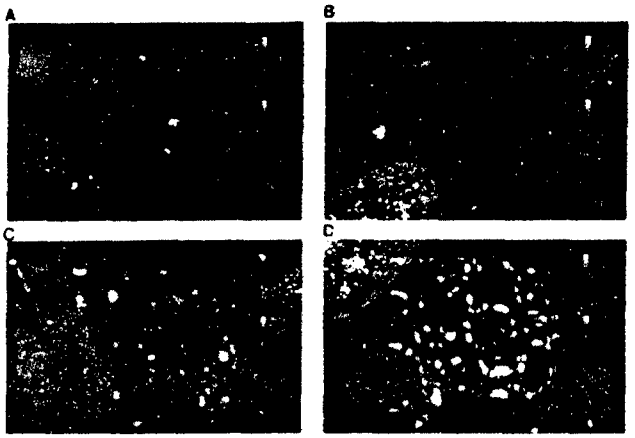

Figure 2. Two dimensional pseudo-color images depicting fluorescence in cells treated with 10 ng/ml TPA for 0, 1, 5 and 10 minutes (panels A, B, C and D respectively).

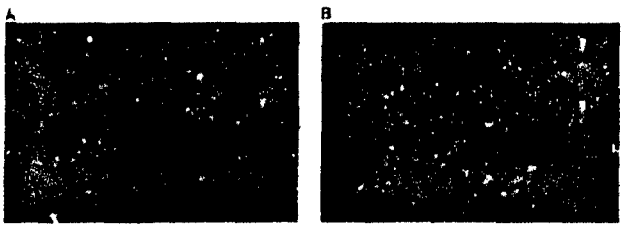

Figure 3. Fluorescent images of rat liver WB cells exposed to no (left panel) or 12.45 mM hydrogen peroxide (right panel).

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Mutation Research, Submitted

In situ (6-4) photoproduct determination by laser cytometry and autoradiography

Toshio Mori[1,3], Tsukasa Matsunaga[1], Chia-Cheng Chang[2], James E. Trosko[2], and Osamu Nikaido[1]

[1]Division of Radiation Biology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920, Japan

[2]Department of Pediatrics and Human Development, Michigan State University, East Lansing, MI 48824, U.S.A.

Running title: In situ (6-4) photoproduct determination

Key words: Monoclonal antibody, (6-4)photoproduct, UV damage, Laser cytometry, In situ determination

[3]To whom all correspondence should be sent:

Dr. Toshio Mori

Division of Radiation Biology

Faculty of Pharmaceutical Sciences

Kanazawa University

Kanazawa 920, Japan

Telefax. 81-762-64-1088

Summary

The UV-induced (6-4) photoproducts and repair in individual human cells were quantitatively determined by using argon-laser imaging microspectrofluorometry or autoradiography with a well-characterized monoclonal antibody against (6-4) photoproducts. (6-4) Photoproduct induction curves were linear as a function of UV dose, using both methods. The formation of (6-4) photoproducts was detected in the cells irradiated with as low as 10 and 25 J/m^2 of UV by autoradiography and laser cytometry, respectively. Normal cells repaired more than 80% of the initial damage within 4 hr post-irradiation. In contrast, almost no repair was observed in xeroderma pigmentosum cells (complementation group A) within 8 hr.

Introduction

Available evidence has not ruled out (6-4) photoproduct as a premutagenic lesion (Franklin and Haseltine, 1986). On the contrary, there is evidence that both cyclobutane pyrimidine dimers and (6-4) photoproducts are responsible for UV-induced mutations in E. coli and in human cells (Brash, 1988). Additional evidence indicating a major biological role for (6-4) photoproducts came from studies of a chemical mutagen-induced revertant of xeroderma pigmentosum cells (x.p., complementation group A) which is normal in repair of (6-4) photoproducts but remains defective in repair of pyrimidine dimers. The revertant was found to be normal in repair replication and UV-induced sister chromatid exchanges and mutations (Cleaver et al 1987, 1988).

Although there is increased interest in (6-4) photoproducts, a sensitive method for detecting (6-4) photoproducts in DNA induced by UV at physiological dose is not readily available except the immunological method (Mitchell and Clarkson, 1984; Eggset et al., 1987; Mori et al., 1988). Besides the advantage of sensitivity, the immunological method also allows researchers to study the distribution of (6-4) photoproducts in individual cells within a tissue or in a population of cultured cells in vitro. For this purpose the antibody used should be very specific for (6-4) photoproducts. The monoclonal antibody specific for (6-4) photoproducts appears to be the best choice for this kind of study.

In this report, we developed a sensitive method to measure the (6-4) photoproducts and their repair in individual human cells in culture by using an argon-laser imaging microspectrofluorometry (Meridian ACAS 470) or autoradiography with a monoclonal antibody against (6-4) photoproducts.

Materials and Methods

Cells and media

Normal human skin fibroblasts (AH) and xeroderma pigmentosum (XP) cells, XP1EH (complementation group A), were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku, Tokyo), supplemented with 10% fetal bovine serum (FBS, M.A. Bioproducts, Walkersville, MD). Another XP cell strain, XP12BE (CRL1223, complementation group A), was obtained from the American Type Culture Collection (Rockville, MD). XP12BE cells were grown in a

modified Eagle's medium (Eagle, 1959) (Earle's balanced salt solution with a 50% increase in all vitamins and essential amino acids except glutamine) and supplemented with nonessential amino acids (100% increase), 1 mM sodium pyruvate, and 10% FBS (Hazleton Research Products, Denver PA). Cells were grown in humidified air with 5% CO_2 at 37°C.

Preparation of a monoclonal antibody specific for (6-4) photoproduct

The details of the establishment of a monoclonal antibody (64M-1) specific for (6-4) photoproducts have been published previously (Mori et al., 1988). Briefly, UV-irradiated single-stranded DNA (20kJ/m^2, UVssDNA) conjugated with methylated bovine serum albumin (mBSA) was injected 3 times into BALB/c mice. Three days after a booster injection of UV-poly(dT)-mBSA, spleen cells were fused with mouse myeloma cells (P3-NSI/1-Ag4-1). Hybridomas secreting antibody recognizing UV-irradiated DNA were screened and the cells in the promising cells were cloned 3 times by limiting dilution. Subsequently, 64M-1, a monoclonal antibody recognizing TT(6-4) photoproducts and TC(6-4) photoproducts was established (Matsunaga et al., In press).

In situ (6-4) photoproduct determination by laser cytometry.

XP12BE cells (2 x 10^5) were directly plated in 35 mm plastic dishes and cultured overnight. After washing with phosphate buffered saline (PBS), cells were irradiated with UV and then fixed with cold methanol : acetic acid (3:1) for 20 min on ice, washed by ethanol and dried. Cells were then treated with 0.07 M NaOH/70%

ethanol for 2 min to denature DNA, followed by washing with PBS (5 times). Cells were incubated with 1% BSA in PBS for 30 min at room temperature to prevent non-specific antibody binding. After washing 5 times with PBS, cells were then incubated with the monoclonal antibody (64M-1) for 30 min at room temperature and washed again with PBS. Cells were then incubated with sheep anti-mouse IgG conjugated with biotin, $F(ab')_2$ fragment (Sigma, St. Louis, MO, final concentration: 25 μg/ml) for 30 min at room temperature and washed five times with PBS. The last step involves the incubation of cells with streptavidin conjugated with FITC (Sigma, St. Louis, MO, final concentration: 10 μg/ml) for 15 min at room temperature, and washing five times with PBS and twice with distilled water. All antibodies and fluorescent chemicals were diluted with PBS containing 0.1% BSA and 0.1% NaN_3. After drying, the cells were covered by a drop of PBS/glycerol (containing 5% wt/vol n-propylgallate), and a clean coverglass with the edges sealed with nail polish. Samples were observed using the Meridian ACAS 470 workstation (Wade et al., 1986, Mori et al., 1989, Meridian Instruments, Okemos, MI). For each treatment, the average fluorescent intensity of about 200 cells in 10 randomly selected ares is presented.

Preparation of ^3H-monoclonal antibody

We followed the method described by Galfre and Milstein (1981). Briefly, 1.2×10^7 hybridoma cells secreting 64M-1 were washed twice with lysine-free DMEM (Nissui Seiyaku, Tokyo) and incubated for 20 hr at 37°C in the incorporation medium: lysine-free DMEM,

3.0 ml; L-[4,5-^{3}H] lysine monohydrochloride (37 MBq/ml, 2.7 TBq/mmole, Amersham, England), 2.16 ml; 10 times Hank's balanced salt solution, 0.24 ml; dialyzed FBS, 0.6 ml. Supernatant was collected and applied on a column containing protein A-Sepharose CL-4B (Pharmacia, Sweden). IgG_{2b} fractions were collected and dialyzed overnight against PBS.

In situ (6-4) photoproduct determination by autoradiography

The method has been described previously (Mori et al., 1988). Briefly, normal skin fibroblasts or XP1EH cells (2 x 10^5) were incubated for 24 hr in a Lab-Tek chamber (No. 4802, Naperville, IL) and washed twice with PBS. Cells were irradiated with various doses of UV at room temperature at a dose rate of 1.13 J/m^2/s. Immediately after irradiation or after post-irradiation incubation, cells were fixed with ice-cold ethanol:acetic acid (3:1) for 20 min at 4°C. Slides with cells were passed through 0.07M NaOH in 70% ethanol for 2 min, rinsed with 70% ethanol and dried. Cells were incubated with PBS containing 10% calf serum (CS) at 37°C for 30 min and subsequently with the ^{3}H-monoclonal antibody (13.9 µg/ml, 3.8 x 10^5 dpm/µg protein) for 30 min. Cells were then serially washed with PBS containing 10% CS, PBS and water. Dried slides were processed for autoradiography using NR-M2 emulsion (Konishiroku Photo Inc., Tokyo). The slides were kept in a freezer at -20°C for exposure for 30 days, developed, fixed and stained with Giemsa solution.

Results

In situ (6-4) photoproduct determination by laser cytometry

Fig. 1 shows the typical fluorescent images of XP cells irradiated with various doses of UV obtained from the ACAS 470 workstation. In the irradiated cells, there was a weak fluorescence on the whole cells. In the cells irradiated with UV, there was a strong fluorescence over the nuclei, in addition to a weak fluorescence over the whole cells. The fluorescent intensities on the nuclei clearly increased with increasing UV doses.

The software associated with the Meridian ACAS 470 workstation allows one to eliminate the background level fluorescence and quantitates the fluorescent intensity per nucleus from the original fluorescent image shown in Fig. 1. Using this method, we measured the fluorescent intensity per cell as a function of UV dose in XP cells (Fig. 2). There was a little background level fluorescence in unirradiated cells and the fluorescent intensity per cell increased linearly with increasing UV doses. We could detect the formation of (6-4) photoproducts in individual cells irradiated with a UV dose as low as 25 J/m^2. The method, however is not sensitive enough to detect (6-4) photoproducts in individual cells induced at lower but biologically significant dose level. To increase the sensitivity, we tried the autoradiography with the ^3H-labelled monoclonal antibody as described in the following.

In situ (6-4) photoproduct determination by autoradiography

Fig. 3 shows the autoradiographs of UV-irradiated and

unirradiated normal human cells treated with ^{3}H-monoclonal antibody. In the unirradiated cells, some background level grains were observed on the whole cells. In the cells irradiated with 50 J/m^2, many grains appeared only on the nuclei.

To quantitate the formation of (6-4) photoproducts in individual cells irradiated with various UV doses, we counted the grain number per nucleus under a microscope. Fig. 4 shows the induction of (6-4) photoproducts in the DNA of UV-irradiated normal and XP cells. The number of grains per nucleus increased linearly with increasing UV doses. As expected, there was no difference in the induction of DNA damage between the two types of cells. Even at as low as 10 J/m^2 of UV dose, there was a significant increase of average grain number per nucleus compared with control cells.

Fig. 5 and 6 show the time course of repair in normal and XP cells after irradiation with 20 and 40 J/m^2 of UV. Normal cells repaired more than 80% of the initial (6-4) photoproducts within 4 hr. On the other hand, XP cells were incapable of repairing (6-4) photoproduct within 8 hr.

Discussion

The results of this study indicate that the two methods, laser cytometry or autoradiography combined with a monoclonal antibody against (6-4) photoproducts, are suitable for measuring (6-4) photoproducts induced by physiological UV doses in individual human cells. The linear (6-4) photoproducts induction curves as a function of UV dose were obtained using both methods. The

formation of (6-4) photoproducts in the cells irradiated with as low as 10 and 25 J/m^2 of UV were detected by autoradiography and laser cytometry, respectively. One third of thymine dimer induction (Mitchell, 1988), 3.2×10^{-19} mole (1.9×10^5/cell) and 7.9×10^{-19} mole (4.8×10^5/cell) of (6-4) photoproducts could be detected by autoradiography and laser cytometry respectively, assuming 10 pg DNA/cell, and 0.0025% of total thymines (unpublished data) and (6-4) photoproduct induction.

Although XP cells could repair almost no (6-4) photoproducts within 8 hr, normal cells repaired more than 80% of the initial damage within 4 hr. These results were quite different from the repair patterns of pyrimidine dimers detected by laser cytometry (Mori et al., 1989), in that normal cells repaired 50% of the initial pyrimidine dimers within 8 hr and 60% at 24 hr after UV irradiation. The fast repair pattern of (6-4) photoproducts in normal cells resembles the results obtained by Mitchell et al. (1985). In their study, the removal of 75% of (6-4) photoproducts was observed within 4 hr in human cells. These results, in combination with the results obtained by Cleaver et al. (1987, 1988), suggest that there might be different short repair processes for (6-4) photoproducts and pyrimidine dimers, in addition to the long common process.

We believe this is the first report of in situ (6-4) photoproduct determination with a well-characterized monoclonal antibody. The monoclonal antibody we used is very specific for (6-4) photoproducts formed in TT or TC sequence (Matsunaga et al.,

In press). Polyclonal antibodies against UV-irradiated DNA do not seem to be suitable for *in situ* determination of a specific damage, because they might contain mixed populations of antibodies recognizing various kinds of damages; i.e. (6-4) photoproducts, pyrimidine dimers and thymine glycol (Mitchell and Clarkson; 1984, Eggset *et al.*; 1987).

Recently, we have established four new monoclonal antibodies specific for (6-4) photoproducts (unpublished data). Preliminary data show that three of them seem to be more sensitive for detection of (6-4) photoproducts than the 64M-1 antibody. We should be able to increase sensitivity for *in situ* (6-4) photoproducts determination using these new antibodies.

Acknowledgements

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Figure Legends

Fig. 1. The typical fluorescent images of XP12BE cells irradiated with various doses of UV obtained from the ACAS 470 workstation. The values shown in the pictures are UV doses (J/m^2).

Fig. 2. The induction of (6-4) photoproducts as measured by the in situ immunofluorescent laser cytometry in XP12BE cells irradiated with various UV doses.

Fig. 3. The autoradiographs of UV-irradiated and unirradiated normal human cells treated with ^3H-monoclonal antibody. The values shown in the pictures are UV doses (J/m^2).

Fig. 4. The induction of (6-4) photoproducts as measured by autoradiography in normal and XP1EH cells irradiated with various UV doses. 100 cells were counted at each point.

Fig. 5. The repair of (6-4) photoproducts in normal and XP1EH cells irradiated with 20 J/m^2 as measured by autoradiography. 100 cells were counted at each point.

Fig. 6. The repair of (6-4) photoproducts in normal and XP1EH cells irradiated with 40 J/m^2 as measured by autoradiography. 100 cells were counted at each point.

Insitu.ms

0
100
25
150
50
200

In Vitro Toxicology, Accepted

Characterization of an In Vitro Human Kidney Epithelial System to Study Gap Junctional Intercellular Communication

B.V. Madhukar, Ph.D.
A.W. de Feijter
C.M. Hasler, Ph.D.
B. Lockwood
C.Y. Oh, Ph.D.*
C.C. Chang, Ph.D.
E. Stanbridge, Ph.D.+
J.E. Trosko, Ph.D.

Department of Pediatrics and Human Development
College of Human Medicine
Michigan State University
East Lansing, Michigan 48824

+Department of Microbiology and Molecular Genetics
College of Medicine
University of California
Irvine, California 92717

*Present Address: Department of Clinical Pharmacology
Flinders Medical Center
Bedford Park, S. Australia 5042

ABSTRACT

In the present study we have investigated if a human cell culture model can be used to study the interaction of xenobiotic chemicals to inhibit gap junctional intercellular communication (GJIC). The G401.2/6TG.1 epithelial cell line derived fro. the kidney of a Wilms' tumor patient exhibited extensive GJIC as measured using FRAP analysis and scrape-loading dye transfer techniques. Several known hepatic tumor promoting chemicals, skin tumor promoter, TPA and polyhalogenated biphenyls were tested for their effect on IC. TPA completely abolished GJIC of these cells at concentrations as low as 0.3 ng/ml and continued to down modulate cell-cell communication for up to 3 days following a single treatment. Among the other chemicals studied, dieldrin, heptachlor and heptachlorepoxide were most effective as inhibitors GJIC; DDT, lindane mirex and Fire Master BP-6 were ineffective. Two fatty acids, linoleic acid and decasohexaenoic acid, were also effective in down regulating cell-cell communication. The data also showed that while TMB-8 antagonized TPA's action on GJIC, dieldrin synergistically interacted to abolish GJIC. The results of the study indicate that the response of these cells may be different from other animal derived cell culture models and suggest the need for using human cell culture models to study toxic interactions of xenobiotic chemicals.

INTRODUCTION

In all metazoans, coordination of cell growth, differentiation and

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Key Words: Gap junctional intercellular communication, cell-cell communication, heptachlor, heptachlor epoxide, TPA, dieldrin, lindane, mirex, PBB's, PCB's, fatty acids, human kidney epithelial cells, free radicals, FRAP analysis, scrape-loading/dye transfer assay.

development, as well as activities of cells within tissues, are mediated by two major forms of intercellular communication of ionic and molecular messages

(Loewenstein, 1981; Potter, 1983; Snyder, 1985; Pitts and Finbow, 1986; Green,1988; Hertzberg and Johnson, 1988). One form of intercellular communication depends on the protein structure, the gap junction, found on the plasma membrane between continguous cells which functions as a channel for ions and molecules of approximately 1000 daltons (Loewenstein, 1979; Evans, 1988). Gap junctions have been shown to be modulated by both endogenous (i.e., hormones, neurotransmitters, growth factors) and exogenous (i.e., biological toxins, drugs, pollutants, nutrients, food additives, pesticides, etc.) agents (Loewenstein and Risinger, 1985; Trosko et al, 1988; Elmore et al, 1987; Neyton and Trautman, 1986). Many cancer cells have reduced or selective gap junctional intercellular communication (Kanno, 1985; Yamasaki and Fitzgerald, 1988). In addition, many chemicals having been shown to be teratogens (Trosko et al, 1982; Welsch and Stedman, 1984), tumor promoters (Yotti et al, 1979; Murray and Fitzgerald, 1979), and neurotoxicants (Trosko et al, 1987) have been linked to their ability to inhibit gap junction function.

To date many techniques, such as electrocoupling (Enomoto et al, 1981), microinjection/dye transfer (Enomoto and Yamasaki, 1984), metabolic cooperation (Yotci et al, 1979; Williams et al, 1981; Kavanagh et al, 1986; Ruch et al, 1987; Jone et al, 1987; Gupta et al, 1985; Hooper, 1981; Davidson et al, 1985; Mosser and Bols, 1982), fluorescence recovery after photobleaching (Wade et al, 1986), and scrape-loading/dye transfer (El-Fouly et al, 1986), have been utilized to measure gap junction function. In addition, many different cell types, strains, and lines have been studied. An emerging picture indicates that not all gap junctions in different cell types respond to the same chemicals in the same manner, possibly due to species, or tissue type receptor differences, as well as differences in gap junction proteins (Saez et al, 1989). While teratogens, tumor promoters and neurotoxicants show species, tissue and developmental stage differences, it would be surprising that a single cell type in vitro system could be predictive of all in vivo conditions (Trosko et al, 1988).

Clearly, if in vitro assays are to be used for risk assessment purposes, as well as for studies of mechanisms of action by which various toxicants work, a series of human epithelial and mesenchymal assays from various organs will eventually be required to minimize the errors of extrapolating data from animal cells to human beings. Consequently, this study was designed to develop a human kidney epithelial cell system to detect chemicals which could modulate gap junction function. The results indicate that this cell line will be an excellent system for further study.

MATERIALS AND METHODS

Cell Culture

The human kidney epithelial cell line G401.2/6TG.1 used in these experiments was originally derived from a Wilms' tumor patient and established as described previously (Weissman et al., 1987). Cells were maintained in D-medium, a modified Eagle's medium containing Earle's balanced salt solution with a 50% increase of vitamins and essential amino acids except glutamine, a 100% increase of non-essential amino acids and 1 mM sodium pyruvate, 5.5 mM glucose, 14.3 mM NaCl and 11.9 mM $NaHCO_3$ (pH 7.3). The medium was supplemented with 5% fetal bovine serum (Gibco Laboratories) and 50 µg/ml gentamicin (Quality Biological, Inc.). The cells were grown at 37°C and 5% CO_2 in a humidified incubator. Under these conditions G401.2\6TG.1 cells had a doubling time of 27 hours.

Chemicals

TPA (Sigma Chemical Co.) was dissolved in ethanol. Dieldrin, p,p'-DDT, lindane, and mirex were generously provided by Dr. Richard E. Leavitt, Pesticide Research Center, Michigan State University. Heptachlor and heptachlor epoxide, 99+% pure were a generous gift of Velsicol Chemical Corporation, Chicago, IL. Aroclor-1254, Firemaster BP-6, were kindly provided by Dr. Matthew Zabik, Pesticide Research Center, Michigan State University. 2,4,5,2',4',5'- and 3,4,5,3',4',5' hexachlorobiphenyl (HCB) and 2,4,5,2',4',5' hexabromobiphenyl (HBB) were purchased from Analabs, North Haven, CN and were 99+% pure. Stock solutions and subsequent dilutions of the test chemicals were made in ethanol or dimethyl sulfoxide (DMSO) and were aded directly to the culture dishes to obtain the required concentrations. Hydrogen peroxide (30% H_2O_2), palmitic acid (PAL, 16:0), linoleic acid (LA, 18:2), linolenic acid (LN, 18:3) and docosahexaenoic acid (DHA, 22:6) were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of fatty acids were dissolved in ethanol and kept frozen under an atmosphere of nitrogen at -20°C. To achieve the desired working concentration of fatty acid, aliquots of stock fatty acids were added directly to complete media supplemented with 5% delipidated serum (Pelfreeze Biologicals) and briefly sonicated immediately prior to treatment of the cells. The final concentration of solvent in the medium was 0.1%. For the scrape-loading/dye transfer (SL/DT) experiments Lucifer Yellow CH (Sigma Chemical Co., MW 457.2) and tetramethyl rhodamine dextran (Molecular Probes, Inc., MW 10.000) were dissolved in phosphate-buffered saline (PBS) at a concentration of 0.5 mg/ml (El-Fouly et al., 1987). For the Fluorescence Redistribution After Photobleaching (FRAP) assay 1 mg of 5,6-carboxyfluorescein diacetate (Molecular Probes, Inc.) was dissolved per ml of ethanol (Wade et al., 1986).

Measurement of Gap Junctional Intercellular Communication (GJIC)

Fluorescence Redistribution After Photobleaching (FRAP). For the FRAP assay, originally described by Wade et al. (1986), G401.2/6TG.1 cells were plated at low density in 35 mm plates. After exposure to the test chemicals the cells were rinsed several times with phosphate buffered saline with Ca and Mg (Ca,Mg-PBS) and maintained in 2 ml of Ca,Mg-PBS during FRAP analysis. Fourteen µl of the dye, 5,6-carboxyfluorescein diacetate were added to each plate. After 15 minutes the plates were rinsed several times with Ca,Mg-PBS, 2 ml of Ca,Mg-PBS were kept on the plates and the cells were subjected to analysis using the Meridian ACAS 470 Workstation (Meridian Instruments, Okemos, MI). Selected cells (5 to 8 per scan) were photobleached and monitored for the return of fluorescence at 5 minute intervals for a total period of 15 minutes (4 scans). The experiments were repeated on 4-5 plates at each concentration or treatment. In each plate a single cell was selected but not photobleached to determine the background decline of fluorescence. Occasionally, an isolated cell was photobleached as a negative control for fluorescence redistribution. Results of the FRAP-experiments were usually expressed as the average (percent) recovery of fluorescence ± standard error of the mean (SEM). While the short-term treatments were performed at room temperature, for long-term exposure, cells were incubated at 37°C and the medium was changed every 2 days followed by addition of fresh test chemical(s).

Scrape-loading/dye transfer (SL/DT). The SL/DT technique was performed as described previously. After treatment with the test chemicals, confluent

cultures in 35 mm plates were rinsed several times with phosphate buffered saline without Ca or Mg (PBS) and two ml of the dye mixture (0.5 mg/ml of each Lucifer Yellow and tetramethyl rhodamine dextran in PBS) was added. Three or four scrape lines were made on the monolayer with a surgical blade. After 3 minutes, the cells were rinsed several times with PBS and examined for dye transfer under a Nikon epifluorescence microscope and photographs were taken. Because of its low molecular weight Lucifer Yellow can be transmitted between adjacent cells via gap junctions, but it does not diffuse through intact plasma membranes. The high molecular weight rhodamine dextran (M.W. 10,000 daltons) however, can neither diffuse through intact plasma membranes nor cross the junctional channels, and therefore serves to identify the primary loaded cells. For the long-term experiments, the medium was changed every 2 days and test chemicals were added. All experiments were repeated at least once.

RESULTS

The human kidney epithelial cell line G401 2/6TG.1 expresses a very high level of gap junctional intercellular communication as shown by both FRAP (Fig. 1) and scrape-loading/dye transfer (Fig. 2) techniques. Fifteen minutes after photobleaching of selected cells, the average recovery of fluorescence in G401.2/6TG.1 cells was 53.3%. In the SL/DT assay the dye, Lucifer Yellow distribution extended 8 to 10 rows of cells beyond the primary recipient cells during the 3 minutes after scrape-loading.

Quantitation of the SL/DT assay was done following two methods: The semiquantitation method of the effect of the chemicals on GJIC was done by assigning an arbitrary scale for communication in control cells and comparing the level of communication after chemical treatment with that of the controls. In the other method a more precise quantitative analysis was done by counting the number of cells with Lucifer yellow in a 5 cm^2 area of the scrape-loading dye transfer picture.

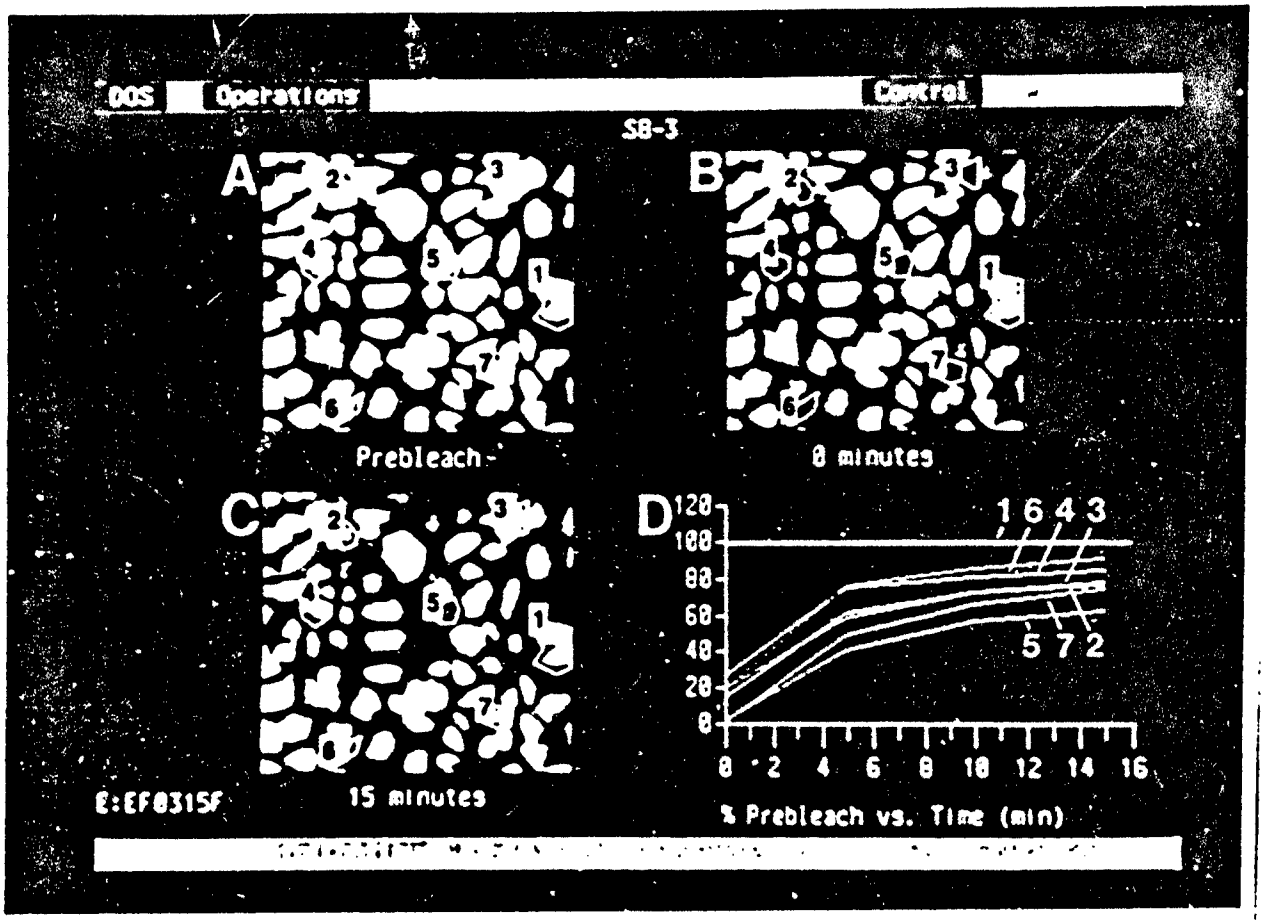

FIGURE 1. Meridian ACAS images of human kidney epithelial G401.2/6TG.1 cells, fluorescently labelled with 5,6-carboxyfluorescein. Digitized pseudo-images (A) prior to, (B) immediately after and (C) 15 minutes after photobleaching of selected cells are displayed. (D) shows a plot in which the percent recovery of fluorescence is expressed in time for each cell selected. Four scans were made at 5 minute intervals after photobleaching. Data were corrected for the background loss of fluorescence in one area (area 1) with two isolated cells. FRAP was performed as described in Materials and Methods.

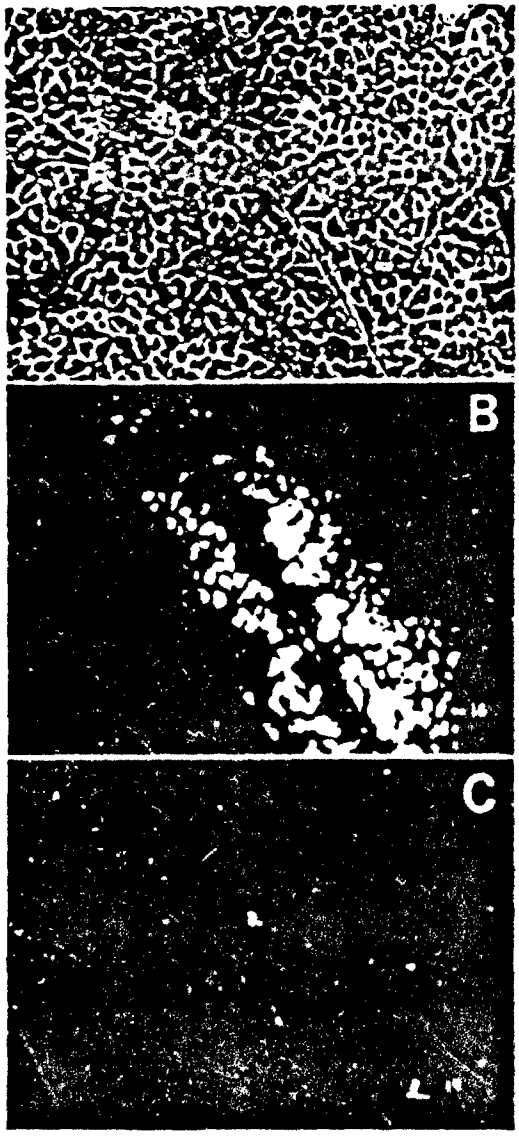

FIGURE 2. GJIC in C401.2/6TG.1 cells as measured by the Lucifer yellow dye transfer technique. Scrape loading of the cells with Lucifer yellow/Rhodamine dextran was done as described. (A) Phase contrast picture of the cells showing normal morphology. Arrow indicates the scrape line for introducing the dye mixture. (B) Lucifer yellow distribution after scrape loading. (C) High mol. wt. Rhodamine dextran is limited to the primary loaded cells only. 300 X

Characterization of TPA-effect on GJIC.

TPA-induced inhibition of intercellular communication in C401.2/6TG.1 cells was characterized using the FRAP assay. Both effects of concentration and exposure time were established. Fig. 3 displays the dose-response curve of TPA

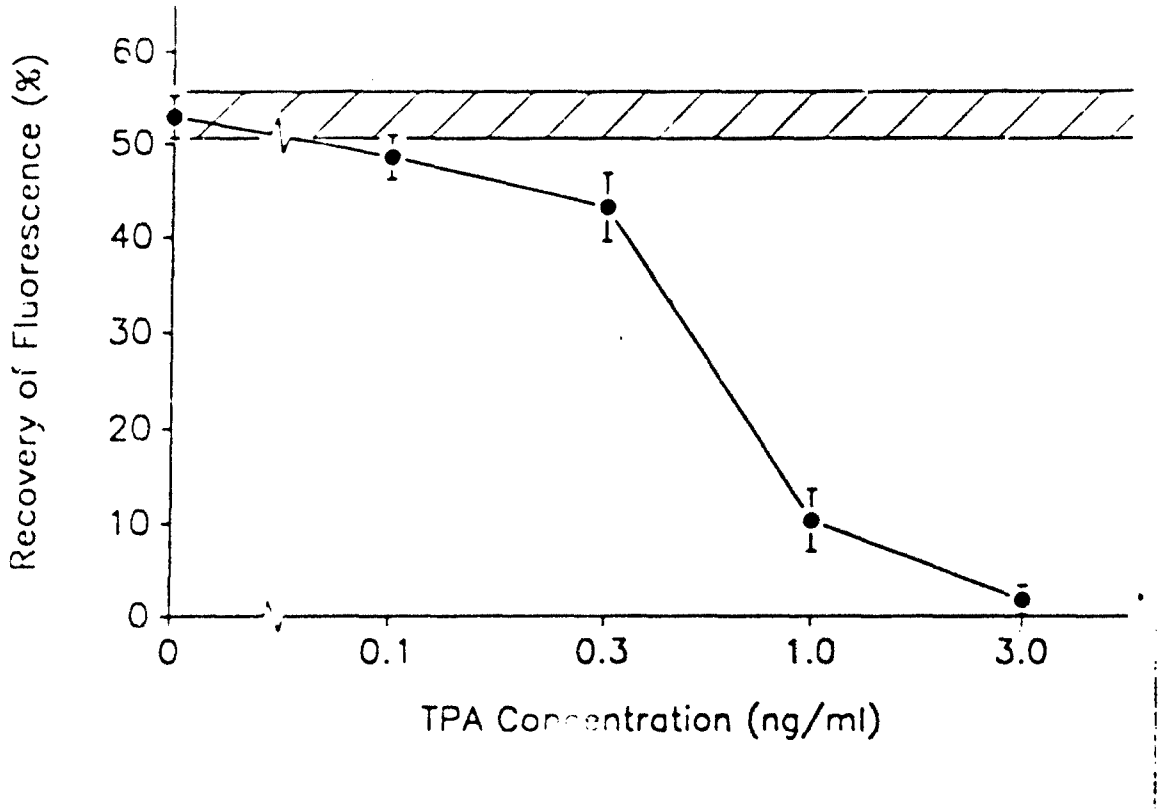

FIGURE 3. Dose-response relationship of TPA-effect on gap junctional communication by FRAP. G401.2/6TG.1 cells were exposed to different concentrations of TPA for 1 hour and fluorescence recovery was determined over a 15-minute period. The shaded area shows the recovery of fluorescence in untreated control cells. Each point in the graph represents the mean recovery ± SEM of 5 experiments.

in G401.2/6TG.1 cells after a 1 hr. exposure. Up to 0.3 ng/ml the compound had no significant effect on the recovery of fluorescence. From 0.3 up to 3.0 ng/ml communication was inhibited in a dose-dependent manner with complete blockage at the highest dose tested. The recovery of fluorescence after different exposure times of G401.2/6TG.1 cells to 3 ng/ml TPA is shown in Fig. 4. When G401.2/6TG.1 cells were continuously treated with TPA, communication was completely blocked up to 1 day. At continued exposure, a time dependent return was observed up to 40% of the control level after 4 days. In ethanol, the solvent for TPA, showed no effect on GJIC up to 4 days of exposure at the concentration applied (Fig. 4).

Characterization of Dieldrin-effect by scrape-loading/dye transfer.

The neurotoxic insecticide, dieldrin was studied for its inhibitory effect on GJIC in G401.2/6TG.1 cells using the dye-transfer assay. Data presented (Fig. 5) show

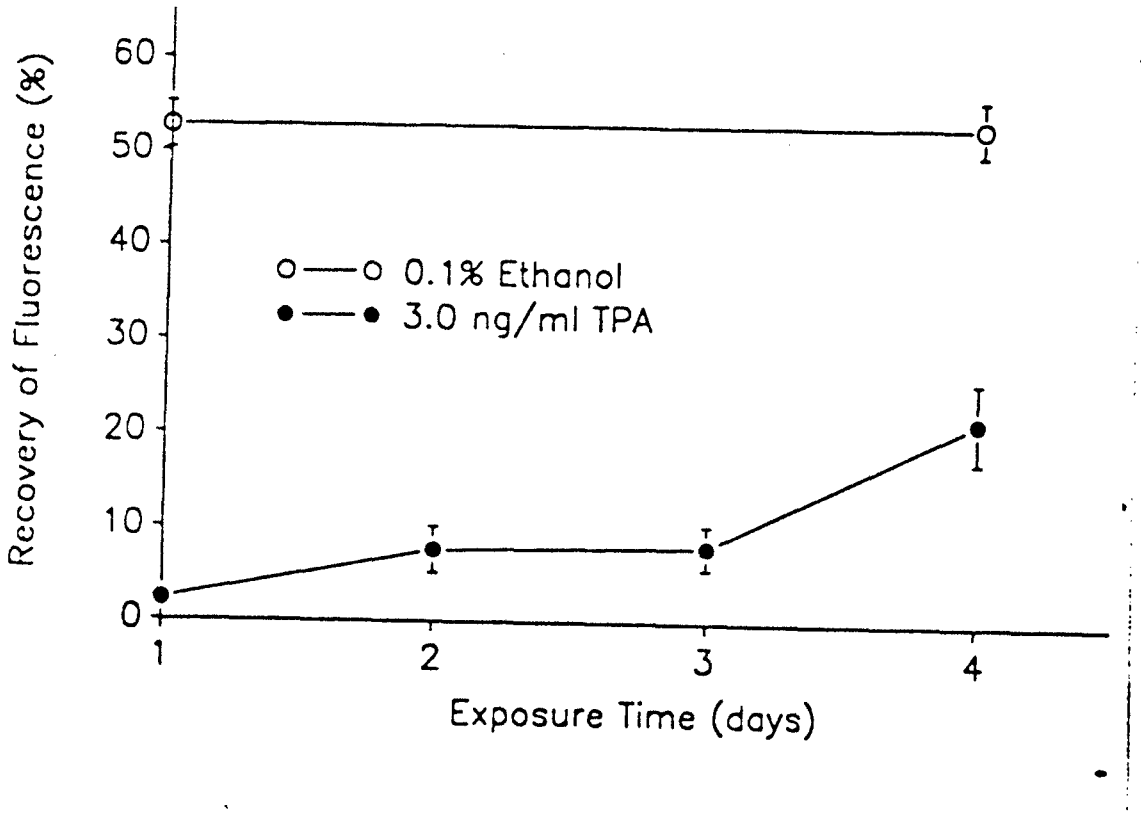

FIGURE 4. Time-response relationship of TPA-effect on gap junctional communication by FRAP. G401.2/6TG.1 cells were treated with 3 ng/ml TPA or the solvent (0.1% ethanol) for 1 to 4 days and fluorescence recovery was determined over a 15-minute period. Each point in the graph represents the mean recovery ± SEM of 5 experiments.

that intercellular communication was inhibited in a time dependent manner. Loss of communication was elicited above 2 μg/ml at one hour after treatment and progresively increased at higher doses up to 10 μg/ul. A progressive loss of GJIC was seen beginning 10 min after treatment with 10 μg/ml dose and was complete at 1 hour. Inhibition sustained for up to 2 hours, following which, the cells gradually regained communication, although the extent of communication was somewhat lower than in controls at the end of 24 hours.

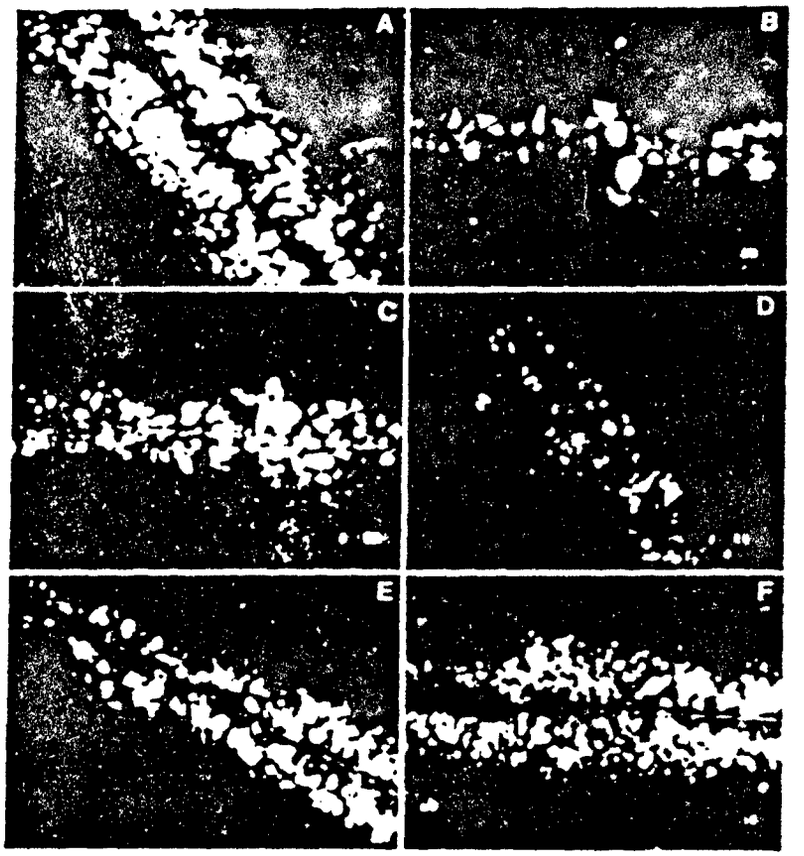

FIGURE 5. Temporal changes in GJIC of G401.2/6TG.1 cells treated with dieldrin. GJIC was determined by the dye transfer method at the required time point after treatment. A. Control. B-F dieldrin (10 μg/ml) at 1, 4, 6, 8, 12 and 24 hrs respectively. 300 X

Effects of other xenobiotics

We have also tested a number of xenobiotic chemicals for their ability to inhibit GJIC using the dye transfer assay. Data presented in Table 1 clearly suggest that there are differences in the action of these agents to down regulate GJIC in G401.2/6TG.1 cells. While the chlorinated insecticides, dieldrin, heptachlor and heptachlor epoxide almost completely abolished communication, p,p' DDT, lindane and mirex were ineffective. The polychlorinated biphenyl (PCB) mixture, Araclor-1254 effectively blocked junctional communication at 10 μg/ml, while the polybrominated biphenyl (PBB) mixture, Firemaster BP-6, did not block GJIC. Interestingly, neither of the hexachlore biphenyl isomers nor the

hexabromobiphenyl tested were able to down regulate GJIC.

Effects of Fatty Acids

Confluent monolayers of G401.2/6TG.1 cells were treated with DHA, LA, Ln, and PAL at concentrations of 25, 100 and 250 uM for various time periods ranging from 5 min up to 24 hr. GJIC was assessed initially utilizing the SL/DT technique. Quantitation of GJIC was done with FRAP analysis. The unsaturated fatty acids of GJIC were done with FRAP analysis. The unsaturated fatty acids DHA and LN significantly inhibited (81%) GJIC at a concentration of 250 uM after 1 hr as assessed by FRAP analysis (Table 2). LA decreased GJIC slightly (25.5%), but fluorescence recovery values in cells treated with LA did not differ statistically from

TABLE 1

Effect of Several Xenobiotics on GJIC in G401.2/6TG.1 Cells[1].

[1]GJIC was measured by scrape-loading/dye transfer technique as described.

[2]Communication in control cells is given an arbitrary scale of ++++, which is equal to the spread of Lucifer yellow into 8-10 rows of cells beyond the primary loaded cells. + indicates significant loss of communication, indicated by spread of Lucifer yellow to 1 layer of cells at the most. The extent of communication was confirmed in 3 different plates in each treatment.

Treatment (ng or μg/ml)	Relative Communication[2]
Control - solvent	++++
TPA - 10 ng/ml	<+
dieldrin - 10 μg/ml	+
Heptachlor - 10 μg/ml	+
p,p'-DDT - 10 μg/ml	+++
Lindane - 10 μg/ml	++++
Mirex 10 μg/ml	++++
Aroclor - 1254 10 μg/ml	+
2,4,5,2',4',5' HCB 10 μg/ml	++++
3,4,5,3',4',5' HCB 10 μg/ml	++++
Firemaster BP-6 10 μg/ml	++++
2,4,5,2',4',5' HBB 10 μg/ml	++++

TABLE 2

Effect of Various Fatty Acids on Dye Recovery in G401.2/6TG.1 Cells[a]

[a]Cells were treated with fatty acids (250 uM) for 1 hr.

[b]% dye recovery (normalizing using a transformation procedure) is expressed as means ± SE for n = 38 (control), n = 7 (LA), n = 9 (DHA), n = 10 (PAL), n = 12 (LN) plates.
[c]Significantly different from untreated controls, $p < 0.05$, Tukey's test.

Treatment	Dye Recovery[b]
Control	50.3 ± 2.5
Linoleic Acid (LA)	37.5 ± 4.5
Linolenic Acid (LN)	9.6 ± 1.4[c]
Palmitic Acid (PAL)	49.6 ± 4.1
Docosahexaenoic Acid (DHA)	9.3 ± 2.5[c]

untreated controls. GJIC in G401.2/6TG.1 cells treated with PAL was similar to that of control cells. The fatty acid-induced inhibition of GJIC by LN and DHA was rapid, occurring within 5 min, and reversible, with GJIC returning to control levels within 1 hr upon removal of fatty acid treatment from cells (data not shown).

Effect of H_2O_2

H_2O_2 concentrations were determined by freshly diluting the 30% stock solution with distilled water and measuring absorbance on a uv/visible spectrophotometer at 240 nm. Exposure of confluent monolayers of SB-3 cells to 100, 250, 500, 1000 and 2000 uM H_2O_2 in serum-free D media for 1 hr had no effect on GJIC. These findings differ with results obtained utilizing liver epithelial WB-F344 cells, (Hasler, unpublished observations) in which H_2O_2 at concentrations of 250 and 500 uM had inhibited GJIC.

Antagonists to chemical inhibition of gap junctional communication.

In a previous study we have reported that in the rat liver epithelial cell line, WB F-344, TMB-8 negated the inhibitory action of TPA on GJIC (Oh et al, 1988). Since the G401.2/6TG.1 cell line appeared to respond differently from the rat liver cell line to the action of xenobiotics, we investigated if TMB-8 would exert the same protective action in G401.2/6TG.1 cells by preventing the down modulatory effect of TPA. As shown in Fig. 6, when the G401.2/6TG.1 cells were pretreated with TMB-8 (50 μM) 10 min prior to treatment with TPA (10 ng/ml), GJIC was only minimally reduced as opposed to complete inhibition by TPA alone. In contrast, TMB-8 appeared to have no effect on dieldrin inhibited GJIC under the experimental conditions used. TMB-8 itself, under these conditions had no effect on GJIC (data not shown). However, the protective action of TMB-8 appeared to be transient under these conditions, as we have noted complete loss

appeared to be transient under these conditions, as we have noted complete loss of communication after 2 hours in cells treated with TMB-8 and TPA.

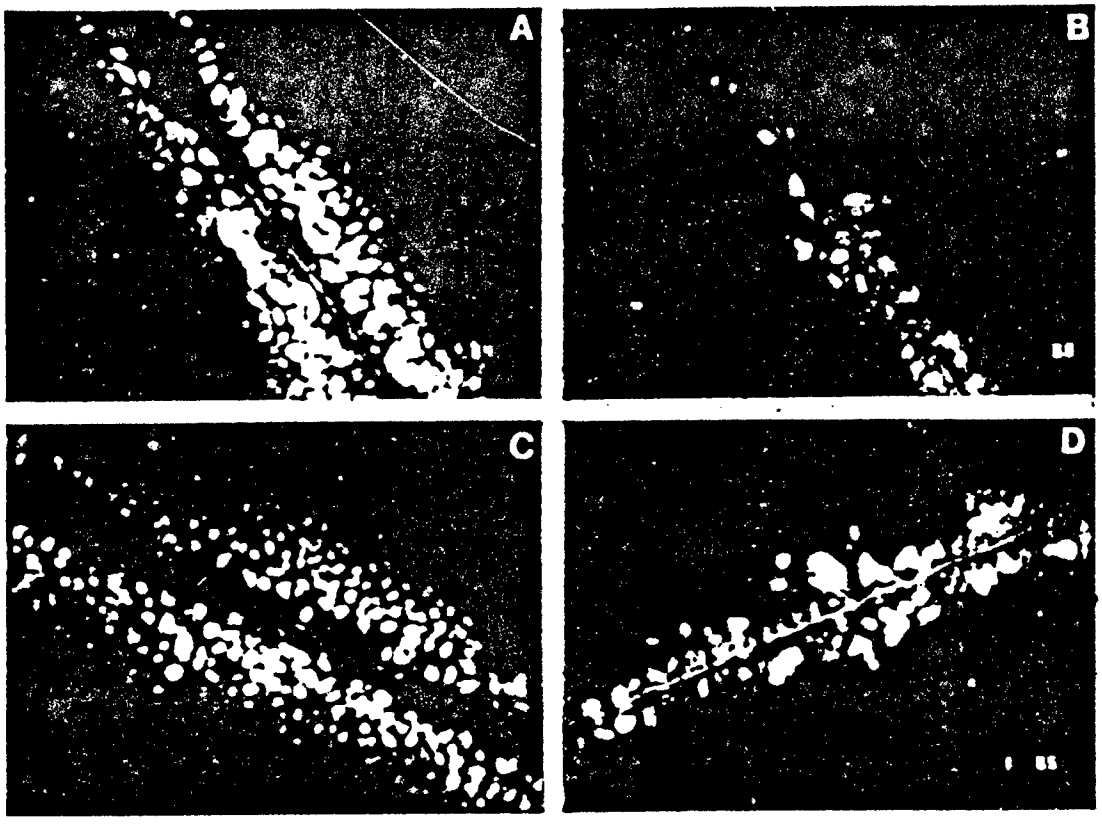

Fig. 6 Antagonistic action of TMB-8 on the inhibition of GJIC by TPA or dieldrin in G401.2/6TG.1 cells. Communication was assayed by Lucifer yellow distribution after scrape loading. Treatment with TMB-8 was done 10 min before adding TPA or dieldrin. A. TMB-8 (50 μM). B. TPA 100 ng/ml. C. TMB-8 + TPA 100. D. TMB-8 + dieldrin (10 μg/ml).

Synergism between TPA and dieldrin.

Synergism between chemicals is a phenomenon by which two mechanistically different chemicals potentiate each others action when added together at levels at which either agent alone has only minimal effect. We have examined if TPA and dieldrin exhibit a synergistic interactions to down-modulate GJIC. As presented in Fig. 7 (A-C) TPA at 1 ng/ml or dieldrin at 1 μg/ml had only a marginal effect in abolishing GJIC under the experimental conditions used. When added together at the same concentrations, under similar conditions, communication was significantly inhibited (Fig. 7D) by these agents. The data thus suggest that these agents exert their inhibitory action of GJIC through divergent mechanisms.

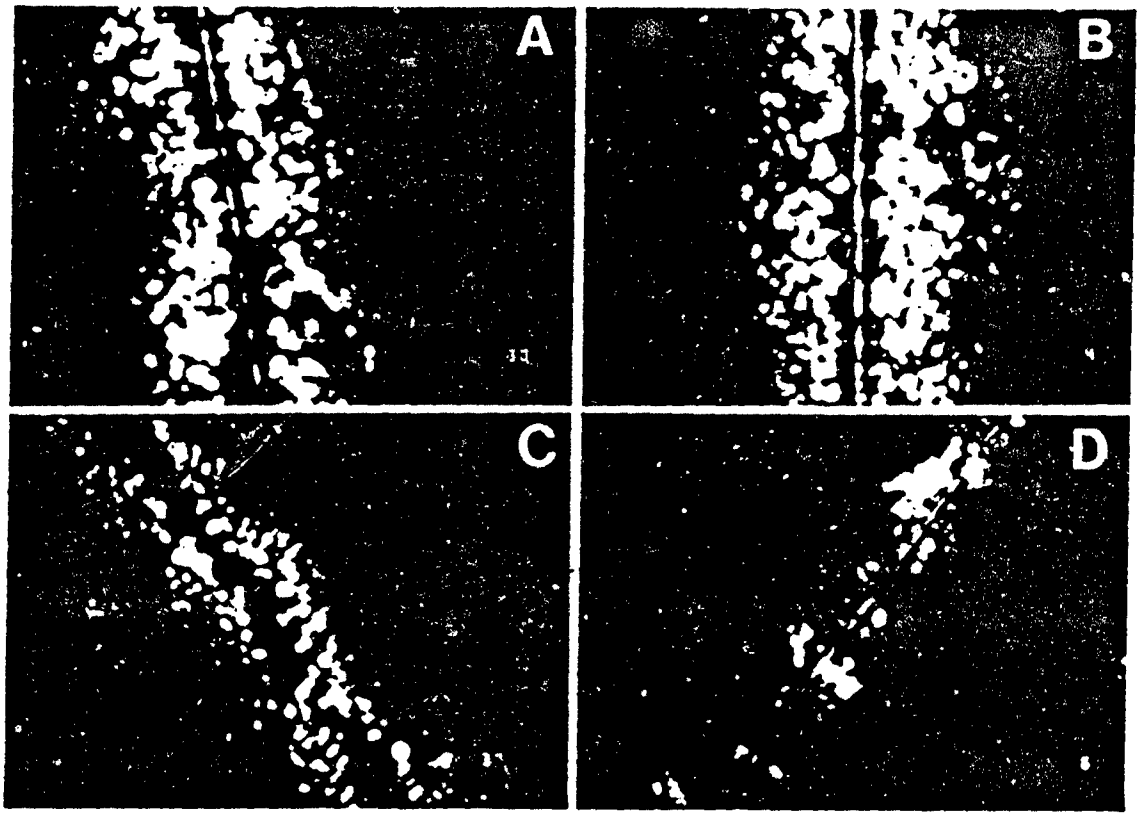

Fig. 7 Synergistic interaction between TPA (1.0 ng/ml) and dieldrin (1 μg/ml) to inhibit GJIC in C401.2/6TG.1 cells. Communication was determined 1 hr after treatment by the dye transfer technique as described. Lucifer yellow distribution in A. Control. B. Dieldrin (1 μg/ml). C. TPA (1.0 ng/ml). D. TPA + dieldrin. 300 X

DISCUSSION

In the present study we have investigated the nature of modulation of GJIC in a human tissue derived cell culture system, with a view to develop a *in vitro* model to study the toxic interactions of xenobiotic chemicals. Clearly, *in vitro*

systems derived from human tissue are advantageous to draw meaningful conclusions on the possible effects of xenobiotic exposure of humans. The G401.2/6TG.1 cell line is derived from a Wilm's nephroblastoma tumor and is of epithelial origin. Since gap junctional intercellular communication has been implicated to have a major role in embryonic development, normal cellular growth and proliferation, perturbations in the normal pattern of GJIC by xenobiotics can be of significant importance in determining the potential toxicity of chemicals to humans. In this context, the G401.2/6TG.1 cell line is an excellent model, since these cells have extensive GJIC and disturbances in the extent of intercellular communication by chemical toxicants can be evaluated with relative ease although the results are not a true replication of the situation with primary cells of human origin. The retropolation of these results should be considered with caution.

The results of the present study clearly suggest that while GJIC in this system is down regulated by xenobiotics, not all the chemicals studied inhibited intercellular communication in these cells. While TPA, a well known tumor promoting agent and a potent inhibitor of gap junctional function, blocked dye transfer of G401.2/6TG.1 cells, there were clear differences in the response of several halogenated hydrocarbon pesticides. Dieldrin and heptachlorepoxide, two neurotoxic insecticides, inhibited GJIC but three other neurotoxic chemicals, DDT, lindane and mirex, did not have any effect. Previous studies from this laboratory, using V79 Chinese hamster lung fibroblasts and WB rat liver epithelial cells, have reported a number of these chemicals which were ineffective in G401.2/6TG.1 cells that have abolished metabolic cooperation and/or dye transfer in these cell lines (Trosko et al, 1987; Suter et al, 1987; Rezebek et al, 1987; Tsushimoto et al, 1987).

An interesting observation in the present study was the difference between the action of TPA and dieldrin in inhibiting GJIC. In a previous study using rat liver epithelial cell system, WB F-344, we noted that TPA's inhibitory action on GJIC was transient while that of dieldrin was sustained for more than 24 hrs (Oh et al, 1988). In contrast, in G401.2/6TG.1 cells, TPA down modulated gap junction function for more than 2 days after a single treatment whereas the effect of dieldrin was reversed as early as 4 hours. It is well known that many of the biochemical effects of TPA are mediated by the activation of a calcium- and phospholipid-dependent protein kinase, protein kinase C (PKC) (Jong and Blumberg, 1989). This kinase is activated upon translocation into the plasma membrane from the cytosol. It is likely that in the case of G401.2/6TG.1 cells, TPA translocated PKC remains plasma membrane bound for a longer period of time than it is in other cell lines including the WB cells (Oh et al, 1988). Indeed, our preliminary observation on the translocation of PKC indicates this to be the case (unpublished observations). While the inhibitory action of TPA on GJIC can be explained by activation and translocation of PKC in G401.2/6TG.1 cells, the mechanism by which the neurotoxic agent, dieldrin, inhibited intercellular communication is less clear. Since increased intracellular free calcium, $(Ca^{++})i$ has been implicated in junctional uncoupling, it is possible that dieldrin treatment might lead to increased $[Ca^{2+}]i$ through inhibition of the Ca^{2+} pump.

In the present study, we have also shown that the unsaturated fatty acids LN and LA differ in their action on GJIC in these cells. In a previous study Aylsworth et al have shown that in Chinese hamster lung fibroblast cell line V79, many of these unsaturated fatty acids have abrogated metabolic cooperation again emphasizing the differences in the response of various cell types to the same chemical(s). Since the unsaturated fatty acids are of considerable health concern their action on GJIC may be significant as a biochemical marker.

In summary, whatever mechanisms might be involved in the uncoupling of gap junctions by xenobiotics, it is clear from our present study that there are

intrinsic differences in the response of various tissue cell types to the inhibitory action of chemicals, thus warranting the need to study more than one cell culture system in order to ascertain the toxic potential of xenobiotics. Moreover, with the demonstration that there is a family of gap junction proteins (see Saez et al, 1989), there is clearly a need to study how specific gap junction proteins might be regulated by cellular responses to different xenobiotics.

ACKNOWLEDGEMENTS

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Corresponding Author

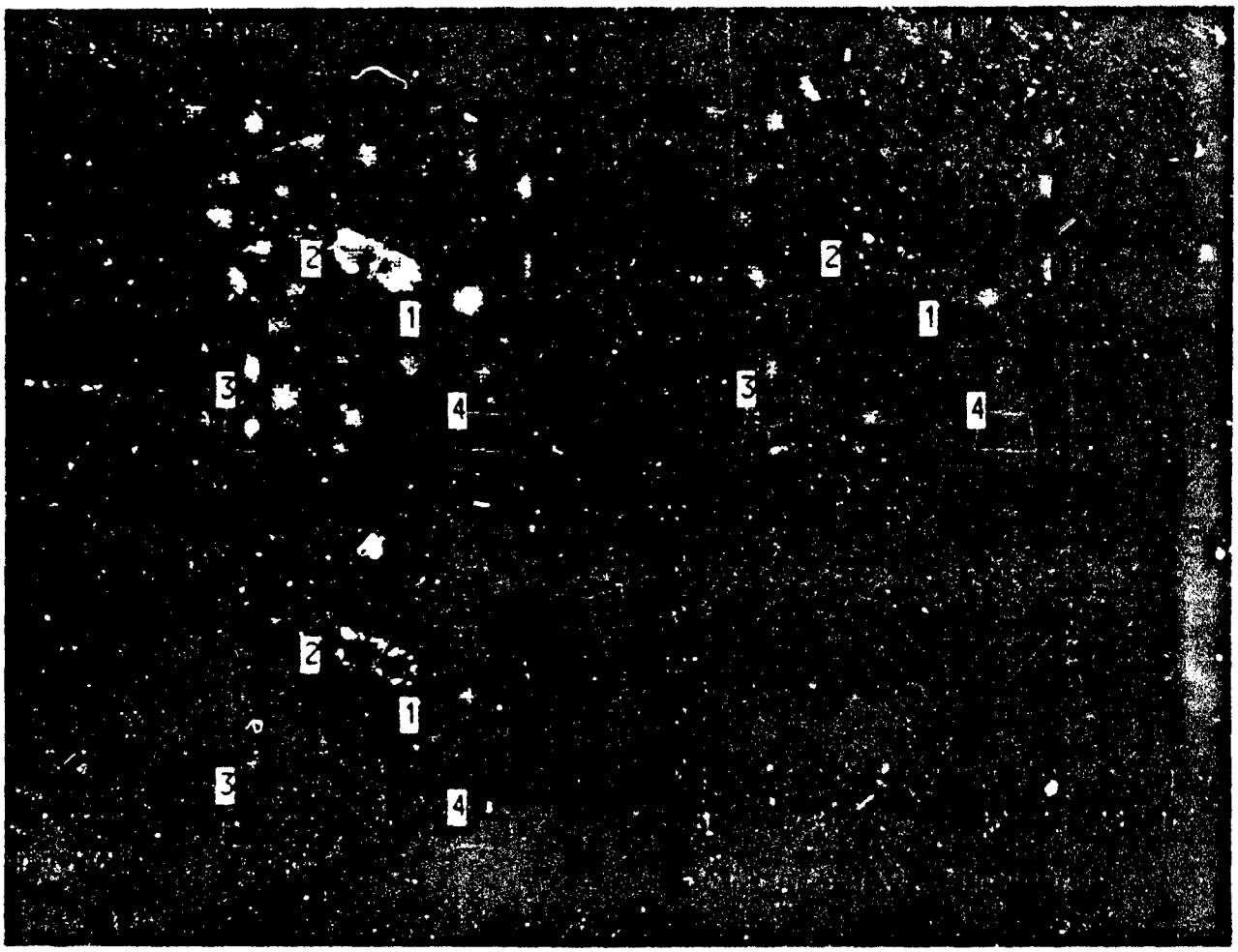
B.V. Madhukar, Ph.D.
Department of Pediatrics/
Human Development
Michigan State University
E. Lansing, Michigan
48824
Tel: 517/353-5431

APPENDIX C

New technique to measure heterologous and homologous cell-cell communication using the ACAS:

1. Actual ACAS-Image data.

2. Abstract.



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Homologous and Heterologous Communication in Co-cultures of Normal and raf-transfected Rat Liver Epithelial Cells. Panels show FRAP analysis, using the Meridian ACAS-570 laser cytometer, of homologous communication between normal RLE cells as indicated by cell #3 and heterologous communication between normal and raf t sformed RLE cells as indicated by cells #2 and #3. V-raf transformed cells ar. loaded with 0.7 um fluorescent beads and then mixed with an excess of normal rat liver epithelial cells. Cells #1 and #2 are two such bead loaded cells which are surrounded by normal RLE cells. Cell #3 is a normal RLE cell. Cell #4 is an unbleached control.

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Gap junctional intercellular communication in raf and raf/myc transformed rat liver epithelial cell lines. G.H. Kalimi[1], J.E. Trosko[1], L.L. Hampton[2], S.S. Thorgeirsson[2], and A.C. Huggett[2]. [1]Michigan State Univ., East Lansing, MI 48824 and [2]National Cancer Inst., Bethesda, MD 20892.

Rat liver epithelial cell lines transformed with v-raf and v-raf/v-myc represent a good in vitro model of tumor progression (Worland et al., 1990, Mol. Carcinogenesis, in press). We studied homologous gap junctional intercellular communication (GJIC) in these cell lines using dye transfer and fluorescence recovery after photobleaching (FRAP) assays, and heterologous GJIC with normal cells using a new bead labelling-FRAP assay. GJIC was found to be reduced in both series of cell lines and showed a good correlation with increasing tumorigenicity. Northern analysis showed reduced levels of connexin 43 in cell lines exhibiting lower GJIC. While raf transformed cell lines showed good heterologous GJIC with normal RLE cells, the raf/myc.transformed cells had complete absence of heterologous communication. These results imply the cooperation of the two oncogenes in abolishing heterologous communication and further suggest the involvement of both homologous and heterologous communication in tumorigenicity of RLE cell lines.

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